

Short Chain Fatty Acids: The Effect on Adipose Tissue  
Metabolism and Function

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## Abstract

Adipose tissue homeostasis is regulated by a combination of extracellular and intracellular signalling pathways. Activation of surface G protein-coupled receptors (GPCR) and insulin receptors, influence the rate of lipolysis within adipocytes, the pathway responsible for triacylglycerol (TAG) breakdown into non-esterified fatty acids (NEFA) and glycerol. A GPCR for short chain fatty acids (SCFA) has been identified on the surface of mature adipocytes and activation of this receptor by SCFA including acetate, butyrate and propionate has resulted in a decrease in lipolysis, measured as a reduction in NEFA and glycerol concentration in the media. However, evidence for a mechanism of action of SCFA within adipocytes has remained unclear. Therefore, this thesis has aimed to better understand the mechanism(s) by which the SCFA, acetate, regulates adipose tissue metabolism and function, and in particular the pathway of lipolysis.

Through the development of experimental methods *in vitro*, it was determined that the dose of isoproterenol, a  $\beta$ -adrenergic receptor activator, required to stimulate lipolysis by 50 % in murine 3T3-L1 adipocytes, was 5  $\mu$ M, and that no interference with metabolic assays was observed in the presence of any potential treatment condition, either in the basal or stimulated state. In 3T3-L1 cells, in the basal state, treatment of cells with the short chain fatty acid acetate (4 mM), significantly reduced lipolysis, as a measure of NEFA and glycerol ( $P = 0.004$  and  $P = 0.020$ , respectively) after a 180 min incubation. Similarly, in the stimulated state, acetate also reduced NEFA significantly ( $P = 0.020$ ), however, glycerol was not reduced ( $P = 0.529$ ) compared with controls. To evaluate whether the metabolic changes in NEFA and glycerol concentration reflected an intracellular change to the pathway of lipolysis, phosphorylation of the key enzyme, hormone sensitive lipase (HSL), was also analysed. It was identified that in the stimulated state, phosphorylation of HSL, at serine residue 563, was reduced by 15 % in the presence of 4 mM acetate, compared with control, complimentary to metabolic data. However, treatment of isolated primary mouse mature adipocytes with acetate did not produce results comparable to those found in the literature. Instead, accumulation of NEFA and glycerol in the media were found to be negligible. Cell viability may have been a limiting factor regarding the outcome of these studies, and therefore development of a protocol to improve cell viability without compromising cell yield

would prove useful. Furthermore, future work should consider the lack of reduction in glycerol in the media in the stimulated state from 3T3-L1 mature adipocytes. This may be accounted for by fatty acid re-esterification, as the NEFA:glycerol ratios were 1.5:1, compared with the expected 3:1 ratio. Similarly, to re-enforce the effects of acetate on stimulated lipolysis, other phosphorylation sites within HSL may be considered, including SER565.

Overall, the results obtained in this thesis demonstrate that in the mature adipocyte cell line 3T3-L1, an increase in the availability of the SCFA, acetate, resulted in a change in the pathway of lipolysis. This was evidenced by a reduction in the phosphorylation of HSL<sub>(SER563)</sub> under sub-maximal stimulation with isoproterenol, similar to levels observed in the presence of insulin. Complimentary to these data, under the same treatment conditions, incubation with acetate also resulted in a small but significant reduction in NEFA concentration in the media.

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## Glossary

AdPLA	Adipose Phospholipase A
ANOVA	Analysis of Variance
AR	Adrenergic Receptor
AT	Adipose Tissue
ATGL	Adipose Triacylglycerol Lipase
AUC	Area under the curve
BCS	Bovine Calf Serum
BSA-FFA	Bovine Serum Albumin-Fatty Acid Free
cAMP	Cyclic Adenosine Monophosphate
CAT1	Carnitine Acyl Transferase 1
CNS	Central Nervous System
CO <sub>2</sub>	Carbon Dioxide
CoA	Coenzyme A
DAG	Diacylglycerol
DB	Digestion Buffer
DMEM	Dulbecco's Modified Eagle Medium
DMEM-PRF	Dulbecco's Modified Eagle Medium-Phenol Red Free
DMSO	Dimethyl Sulfoxide
EDTA	Ethylenediaminetetraacetic acid
FBS	Fetal Bovine Serum
GAPDH	Glyceraldehyde 3-phosphate Dehydrogenase
GDH	Glycerol Dehydrogenase
GFP	Green Fluorescent Protein
GI	Gastrointestinal Tract
GLUT4	Glucose Transporter Type 4
GPCR	G Protein-Coupled Receptor

GPR43	G Protein-Coupled Receptor 43
H <sup>+</sup>	Hydrogen
HBSS	Hanks Balanced Salt Solution
HCl	Hydrogen Chloride
HDL	High Density Lipoproteins
HEPES	Hydroxyethyl Piperazineethanesulfonic Acid
HRP	Horse Raddish Peroxidase
HSL	Hormone Sensitive Lipase
IBMX	Isobutylmethylxanthine
IR	Insulin Receptor
IRS-1	Insulin Receptor Substrate-1
ISO	Isoproterenol
K <sub>2</sub> HPO <sub>4</sub>	Potassium Phosphate Dibasic
KCl	Potassium Chloride
KH <sub>2</sub> PO <sub>4</sub>	Potassium Phosphate Monobasic
KRB	Krebs Ringer Buffer
KRBHA	Krebs Ringer Buffer HEPES Albumin
LCFA	Long Chain Fatty Acid
LDL	Low Density Lipoproteins
MAG	Monoacylglycerol
MAGL	Monoacylglycerol Lipase
MCFA	Medium Chain Fatty Acid
NA	Nicotinic Acid
Na <sub>2</sub> HPO <sub>4</sub>	Sodium Phosphate Dibasic
NaAc+Ins.	Sodium Acetate + Insulin
NaCl	Sodium Chloride
NAD <sup>+</sup>	Nicotinamide Adenine Dinucleotide
NADH	Nicotinamide Adenine Dinucleotide + Hydrogen

NaOH	Sodium Hydroxide
NEFA	Non Esterified Fatty Acid
NETO	Norepinephrine Turnover
PAT	Perilipin Adipophilin TIP47
PBS	Phosphate Buffered Saline
PDE3B	Phosphodiesterase-3B
PDH	Pyruvate Dehydrogenase Complex
PG	Prostaglandin
PG/ED	Perigonadal/Epididymal
pHSL	phosphorylated Hormone Sensitive Lipase
PI3-KINASE	Phosphoinositol-3-Kinase
PKA	Protein Kinase A
PKB	Protein Kinase B
PMSF	Phenylmethanesulfonyl Fluoride
PRV	Pseudorabies Virus
PSNS	Parasympathetic Nervous System
PVDF	Polyvinylidene Fluoride
SC	Subcutaneous
SCFA	Short Chain Fatty Acids
SDS-PAGE	Sodium Dodecyl Sulphate – Polyacrylamide Gel Electrophoresis
SE	Standard Error of the Mean
SNS	Sympathetic Nervous System
SVC	Stromal Vascular Cell
TAG	Triacylglycerol
TBS	Tris Buffered Saline
TBS-T	Tris Buffered Saline – Tween 20
TCA	Tricarboxylic Acid Cycle
TDB	Tissue Digestion Buffer

VH	Ventromedial Hypothalamus
VLDL	Very Low Density Lipoproteins
WAT	White Adipose Tissue
$\beta$ -AR	Beta-Adrenergic Receptor

# **Chapter 1**

## **Introduction**

## **Whole body metabolism**

In the Scottish Health Survey 2012 (Scottish-Government, 2013) it was reported that over 60 % of men and women living in Scotland were recognised as either overweight or obese, with ~ 30 % having a body mass index (BMI) of  $\geq 30 \text{ kg/m}^2$ . Correlations also exist between overweight/obesity and the co-morbidity of cardiovascular disease, hypertension and Type II diabetes (Keenan et al., 2011). Therefore, there is a clear social and economic benefit for reducing the rate of overweight/obesity and related diseases in the UK and elsewhere. However, in order to achieve this reduction, there is a need to better understand the underlying mechanisms involved in adipose tissue (AT) metabolism and function. The lipolytic cascade involved in the breakdown of lipids in AT is regulated by both  $\beta$ -adrenergic receptor ( $\beta$ -AR) activation and insulin sensing. The converging step, linking both pathways to lipolysis, occurs via the abundance of cyclic adenosine monophosphate (cAMP) within adipocytes. An increase in available cAMP can occur through activation of stimulatory G protein-coupled receptors ( $G\alpha_s$ ), resulting in an increase in non-esterified fatty acid (NEFA) and glycerol release. Conversely, activation of the insulin receptor which is known to reduce cAMP to 5'AMP via phosphodiesterase 3B (PDE3B), results in a reduction in overall lipolysis. Similarly, activation of inhibitory G protein-coupled receptors ( $G\alpha_i$ ), also reduce the availability of cAMP, however, through down regulation of adenylate cyclase. Proteins involved downstream of cAMP sensing within lipolysis, including hormone sensitive lipase (HSL), can be studied for changes in phosphorylation, as a marker for intracellular lipolysis. Identification of changes to the phosphorylation of HSL would ensure lipolysis is directly affected without discriminating between activation sites, including G protein-coupled receptors (GPCR) or insulin receptors. Further to this, measurement of metabolic compounds such as NEFA, glycerol and intracellular triacylglycerol (TAG) would also complement these findings.

Obesity has been documented as an epidemic found predominately in the developed world for over 20 years (WHO, 1990), however, associated diseases, such as Type II diabetes have been investigated for over 70 years (Himsworth, 1939). More recently, studies have focused on understanding the mechanism of action relating diet and nutrition to metabolic disorders. Initial links between the regulation of glucose and fatty acid utilisation were first published in 1963 by Randle et al. (1963). In this paper, Randle and colleagues (1963) summarised that a cyclical process affected both glucose availability/metabolism and free fatty acid production/oxidation and was termed the

glucose fatty acid cycle. The publication included data showing how an increase in fatty acid oxidation impacted on glucose phosphorylation, glycogen metabolism, glycolysis and pyruvate oxidation in rat and human models, using examples from heart, diaphragm muscle and liver, both *in vivo* and *in vitro*. This initial hypothesis in 1963 has also been verified by others, as Nuutila et al. (1992) published data in which an increase in free fatty acid oxidation resulted in a decrease in whole body glucose uptake in humans. An increase in fatty acid oxidation contributed to a decrease in whole body glucose uptake as measured by the decrease in glucose uptake in femoral and arm muscle tissue. Also, glucose uptake was found to be decreased in the heart muscle; however, this decrease did not contribute significantly to the overall decrease in whole body glucose uptake (Nuutila et al., 1992). The mechanisms involved in this cycle are complex; however the importance of the pyruvate dehydrogenase complex (PDH) has shed light on these interlinking metabolic pathways. When activated (de-phosphorylated), the PDH complex catalyses the oxidative decarboxylation of pyruvate to acetyl-CoA, linking glycolysis and energy storage with energy availability and free fatty acid oxidation (Holness and Sugden, 2003), as shown in Fig. 1.1.

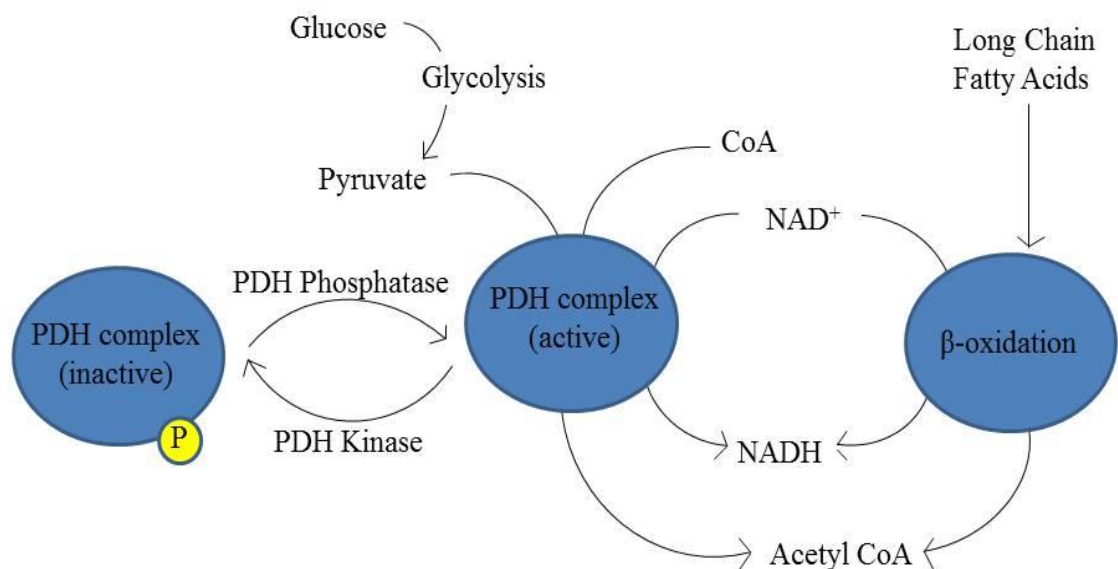


Fig 1.1 PDH complex adapted from Holness and Sugden (2003). Interaction between long chain fatty acid  $\beta$ -oxidation and glycolysis through the PDH complex. PDH, Pyruvate Dehydrogenase;  $\text{NAD}^+$ , Nicotinamide Adenine Dinucleotide; NADH, Nicotinamide Adenine Dinucleotide + Hydrogen; CoA, Co Enzyme A.

Since 1963, further experiments have been conducted to ascertain the molecular basis of these interacting pathways, including a complementary mechanism to the glucose fatty-

acid cycle, reviewed by Frayn (2003). The mechanism proposed by McGarry et al. (1977) inferred inhibition of carnitine acyl transferase 1 (CAT1) by malonyl CoA, which occurred due to excess citrate moving out into the cytosol of liver cells. The test involved the long chain fatty acid (LCFA) and the medium chain fatty acid (MCFA), oleic and octanoic acids, respectively. A decrease in the level of LCFA but no change in the level of MCFA in the presence of malonyl CoA, allowed for the hypothesis that one or more of the LCFA transport proteins must be affected. As malonyl CoA would not be able to make contact with the inner mitochondrial transport machinery, it was proposed that CAT1 must be involved in the decrease of fatty acid oxidation in liver cells. The specificity of malonyl CoA to inhibit CAT1 was elucidated using other CoA-derivatives and intermediates from processes, such as the tricarboxylic acid cycle and from glycogen-pyruvate conversions. No other metabolites were able to produce the same level of reversible inhibition as malonyl CoA (McGarry et al., 1977). Evidence for CAT1 involvement in a glucose induced reduction in fatty acid oxidation have also been identified in other tissues, including muscle, as reviewed by Zammit (1999). These data demonstrate that the glucose fatty acid cycle sustains the homeostatic balance of energy availability within the body. However, weight gain and obesity can ensue if increased calorie intake outweighs energy expenditure.

### **Nutrient availability**

Contributing on average 45 - 65 % of the recommended daily intake of calories, carbohydrates account for a large portion of ingested food on a daily basis (NHS, 2010). Of this, increases in non-digestible carbohydrates, including dietary fibre, such as resistant starch and lactulose (Puupponen-Pimiä et al., 2002), have led to increased levels of fermentation in the gut, and an increase in the production of the short chain fatty acid (SCFA) acetate as a consequence (Ferchaud-Roucher et al., 2005, Macfarlane and Macfarlane, 2003). Alcohol is also recognised as a source of increased SCFA availability following its metabolism in the liver as described by Siler et al. (1999). Nilsson and Belfrage (1978) compared ethanol, acetate and acetaldehyde availability in isolated rat adipocytes and reported that increased acetate, the metabolised form of ethanol, resulted in a decrease in the release of glycerol (measure of lipolysis) by 25 - 70 %. However, the decrease in glycerol production was only observed in the presence of the  $\beta$ -AR stimulator norepinephrine. It should also be noted that the concentrations of acetate used in this study were supra-physiological (0 – 10 mM), compared with the



concentration of acetate released from the liver, after crossing the gut lumen ( $17.3 \pm 7.3$  mmol kg bodyweight<sup>-1</sup> h<sup>-1</sup>). SCFA are typically categorised as having an aliphatic tail of less than 6 carbon atoms, the most common of these being acetate (C2), propionate (C3) and butyrate (C4). The presence of increased SCFA availability in humans has been linked to diet. Studies carried out before 2000 published data on the production of SCFA, their interaction with the gut wall and the effect they had on particular gut related diseases. Whereas over the last 10 years studies have tried to ascertain the fate of SCFA after absorption through the large intestine gut wall. Information available on SCFA after crossing the gut lumen barrier was restricted, as procedures to measure portal and hepatic vein SCFA levels were invasive. A recent study by Bloemen et al. (2009), and the first reported study of portal blood SCFA concentration by Peters et al. (1992) provided a greater insight into where these important fatty acids end up after crossing the gut membrane. It was reported that SCFA could reach the portal vein as quickly as 15 minutes after introduction, illustrating the speed of colonic fermentation and gut absorption (Peters et al., 1992). Recent studies have included measurement of hepatic SCFA levels which were not previously assessed, and clarified our understanding of the fate of SCFA after crossing the gut barrier by describing that propionate and butyrate can become potentially toxic in higher levels (Tedelind et al., 2007, Manns and Boda, 1967) as cited by Bloemen et al. (2009), and are therefore rapidly cleared from the circulation and metabolised by the liver. Propionate, butyrate and acetate were metabolized in the liver, however, roughly 70 % of 24 g administered ethanol was released as acetate by the liver, into the circulation acting on other tissues, including AT and muscle tissue at a concentration of  $47.5 \pm 2.5$  mmol · kg<sup>-1</sup> · min<sup>-1</sup> (Siler et al., 1999). It was identified that the G protein-coupled receptor GPR43 was specific for SCFA (Le Poul et al., 2003), with receptors present in a number of tissues, although in this particular study GPR43 was not found in adipose tissue. However, since 2003, the presence of GPR43 in adipose tissue depots including subcutaneous and epididymal has been identified. Other GPCR sensitive to agonist with small carboxylic tails have also been found in adipose tissue, including the prostaglandin E2 (PGE2) receptor EP3 and nicotinic acid receptor HM74A. The natural ligand for HM74A, however, remains elusive, as physiological concentrations of nicotinic acid are unable to induce a response. (Jaworski et al., 2009, Zhang et al., 2005). Furthermore, activation of GPR43 with SCFA have been found to promote both lipogenesis (Hong et al., 2005) and inhibit lipolysis (Ge et al., 2008). These diverse findings surrounding the effect of SCFA on

AT metabolism demonstrate a need to better understand the mechanism of action involved.

### **Adipose tissue homeostasis: Intracellular and extracellular signalling**

#### **Adipose tissue metabolism**

Regulation of AT encompasses the intricate balance between breakdown and synthesis of triacylglycerol (TAG), for storage, or as an energy source depending on the nutritional status of the mammal. Therefore lipolysis and lipogenesis (Fig. 1.2 and Fig. 1.3) are tightly controlled by a number of signals including but not limited to second messengers, hormones and adipokines (Jaworski et al., 2007). Literature reviews of these pathways, including the interaction between lipolysis and the metabolic syndrome (Langin, 2006), as well as a newly discovered protein, central to the control of lipolysis (Villena et al., 2004), are continually up-dating our understanding of adipose tissue homeostasis.

#### **Intracellular signalling**

Since identifying the existence of adipose triacylglycerol lipase (ATGL/Desnutrin) in 2004, for the hydrolysis of TAG into diacylglycerol (DAG) (Villena et al., 2004, Zimmermann et al., 2004, Jenkins et al., 2004) and a greater understanding of fatty acid re-esterification (Jensen et al., 2001), and TAG turnover as reviewed by Reshef et al. (2003), it has now been established that AT is much more metabolically active than previously reported, and the process of lipid metabolism is more complex. As AT is the main storage site for fat consumed in the diet, the process of lipolysis is responsible for mobilising TAG within lipid droplets located within individual adipocytes. The process utilizes three lipases sequentially hydrolysing fatty acids from the TAG backbone. It was originally hypothesized that HSL was responsible for the first two hydrolysis steps, however it is now widely accepted that ATGL hydrolyses the first free fatty acid. This lipase was found to be more potent than HSL at hydrolysing TAG into DAG, and unlike HSL, which required phosphorylation by PKA to become active, ATGL did not (Villena et al., 2004, Zimmermann et al., 2004, Jenkins et al., 2004). Phosphorylation of HSL by protein kinase A (PKA) stimulates pHSL translocation to the lipid droplet surface where it hydrolyses DAG into MAG, releasing one free fatty acid (Fig. 1.2), finally leaving monoacylglycerol lipase (MAGL) to cleave the final fatty acid from the

glycerol backbone (Duncan et al., 2007). Although the proteins involved in TAG hydrolysis have now been identified, the exact mechanism in which TAG is stored in lipid droplets, and the method by which lipases access these stores has, however, never been fully understood.

Individual adipocytes store TAG in monolayer lipid droplets, however, the way in which these droplets are generated, mature and increase in size still needs further investigation (Walter and Farese Jr, 2009). Several mechanisms for the growth of lipid droplets have been proposed, including influx of TAG instead of droplet to droplet fusion, and also the involvement of Caveolin-1 (Kuerschner et al., 2008, Cohen et al., 2004).

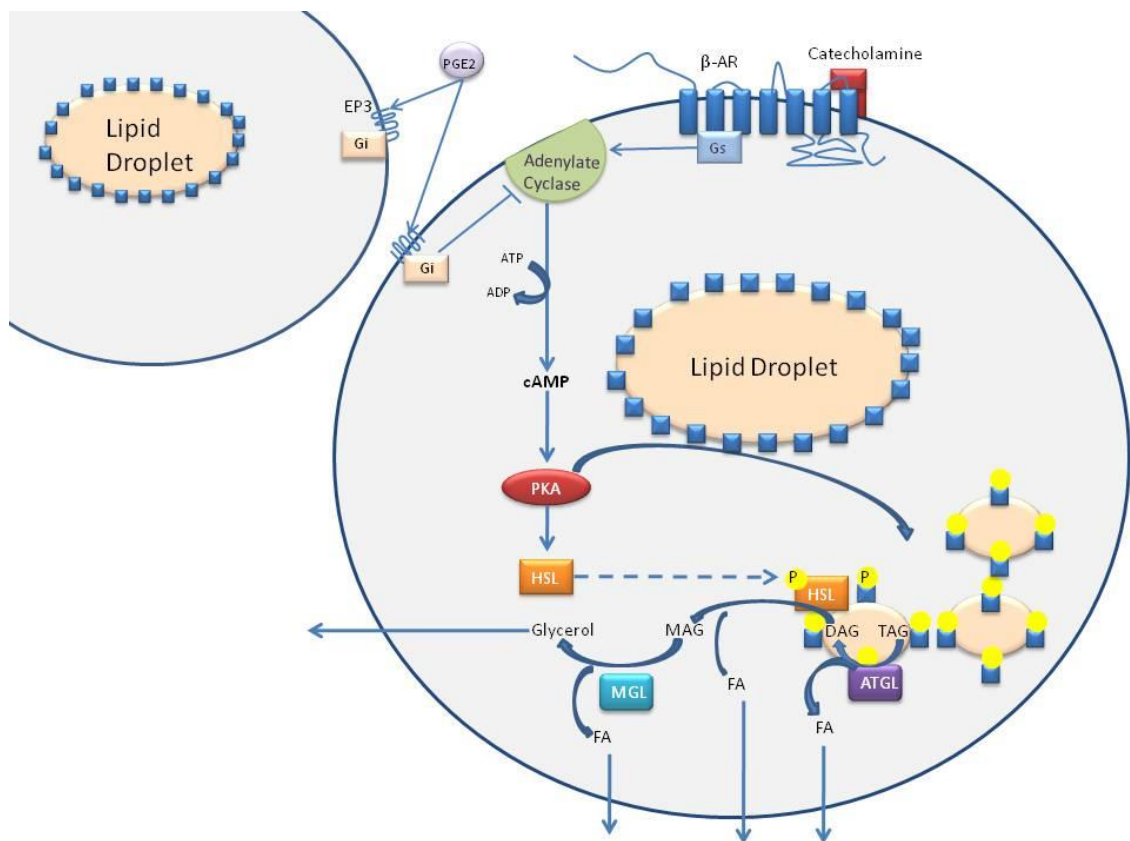


Fig 1.2 Adapted from Marcinkiewicz et al. (2006), Ahmadian et al. (2009). Pathway of lipolysis: Activation of  $G_s$  or  $G_i$  G protein-coupled receptors via  $\beta$ -AR receptors, and the subsequent lipolytic pathway.  $\beta$ -AR, Beta Adrenergic Receptor;  $G_i$ , G alpha inhibitory protein;  $G_s$ , G alpha stimulatory protein; PGE<sub>2</sub>, Prostaglandin E<sub>2</sub>; PKA, Protein Kinase A; HSL, Hormone Sensitive Lipase; ATGL, adipose triacylglycerol lipase; TAG, triacylglycerol; DAG, diacylglycerol; MAG, monoacylglycerol; FA, fatty acids.

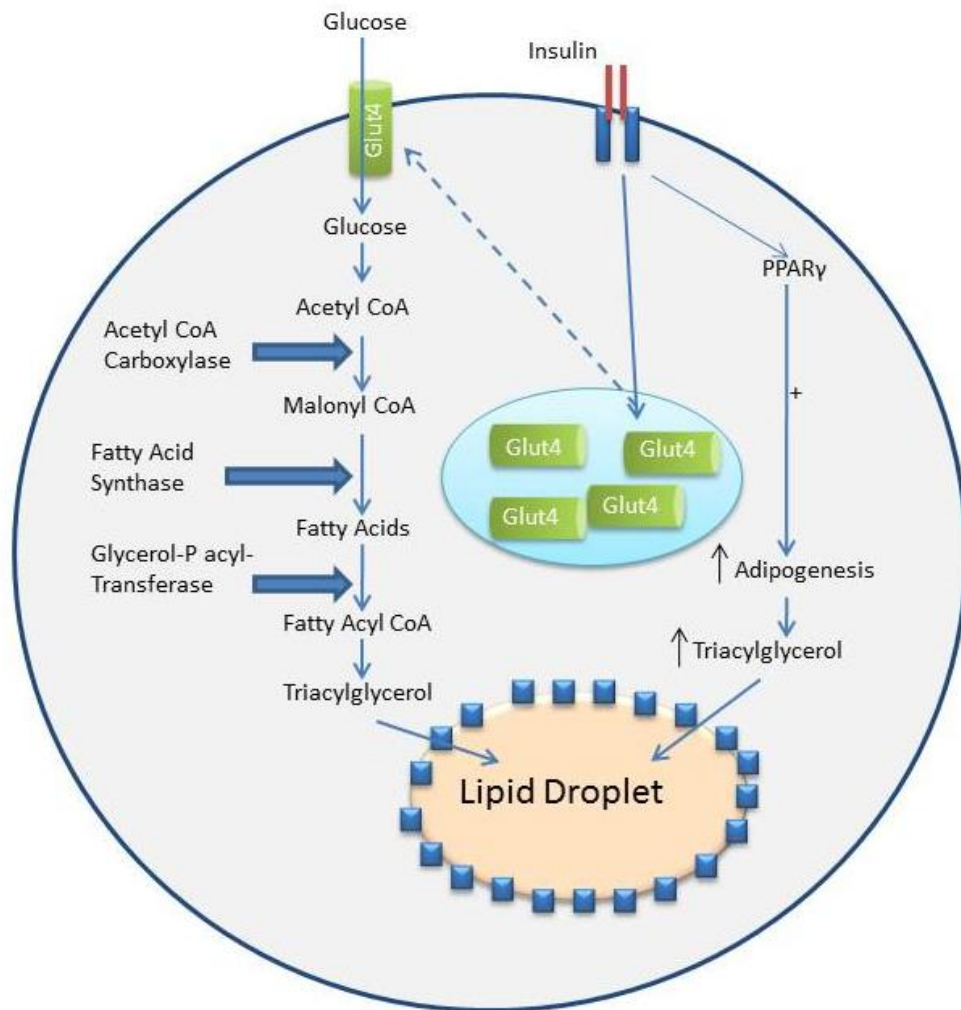


Fig 1.3 Pathway of lipogenesis: Insulin receptor signalling and Glut4 translocation from an intracellular store followed by triacylglycerol synthesis and lipid droplet formation. Glut4 Glucose receptor 4, PPAR $\gamma$  Peroxisome proliferator-activated receptor gamma.

It has been shown that Caveolin null perilipin A mouse embryonic fibroblast (peri MEF) do not build up the same volume of lipid in their lipid droplets compared to wild type mammals to a significant extent. Perigonadal AT isolated from the Caveolin-1 null mice also had a decreased response to lipolytic agonists showing that the decrease in lipid volume due to a lack of Caveolin-1 in the lipid droplet membrane also affected adipose tissue metabolism (Cohen et al., 2004). However, the mechanism by which lipid droplets are formed in the first place remains elusive. The most common assumption is monolayer bulging and budding from the endoplasmic reticulum (ER). The presence of lipid droplets within adipocytes has been noted for many years, however, their complexity has only come to light over the last decade (Brasaemle, 2007, Londos et al., 2005). The main family of proteins associated to the lipid droplet

membrane surface has been identified as the PAT family, consisting of Perilipin, Adipophilin, TIP47 and two others, OXPAT and S3-12. Brasaemle et al. (2004) identified that perilipin, S3-12 and TIP47 localise to the lipid droplet surface, under both basal and stimulated lipolysis in 3T3-L1 cells, whereas adipophilin was selectively localized to the lipid droplet of stimulated cells. It has been hypothesized that perilipin surrounds the lipid droplet creating a 'scaffolding-like' structure allowing access by lipases to the lipid surface only when the perilipin coat is phosphorylated (Brasaemle, 2007). One hypothesis proposes, after phosphorylation of the perilipin coat by PKA, that the lipid droplet breaks down into smaller mini droplets, increasing the surface area of the droplet in contact with the surrounding lipase and therefore allowing the start of TAG hydrolysis (Marcinkiewicz et al., 2006) (Fig 1.2). However, as lipolysis does not function unrestrained, in opposition to an increase in lipolysis and subsequent TAG hydrolysis, activation of the insulin receptor (IR), results in a reduction in lipolysis. As shown by Berggreen et al. (2009), a converging step within the pathway of lipolysis has been identified, where insulin receptor activation resulted in activation of PKB, and subsequent reduction in glycerol release from rat and 3T3-L1 adipocytes. Previously, it has been shown that PKB mediates the activation of PDE3B, the enzyme responsible for converting cAMP to 5'AMP (Kitamura et al., 1999).

After a meal, blood glucose rises, closely followed by a rise in plasma insulin released from pancreatic  $\beta$ -cells. In adipocytes, the binding of insulin to its receptor, leads to a decrease in overall lipolysis and therefore a decrease in NEFA and glycerol release. In metabolic disorders, such as Type II diabetes, insulin sensing becomes impaired, leading to changes that include hyperglycaemia (Rigalleau et al., 2010) and hyperinsulinemia, as reviewed by Shanik et al. (2008). The signalling pathway involving insulin is complex, as evidence for both PKB dependant (Kitamura et al., 1999) and independent (Choi et al., 2010) regulation of PDE3B have been proposed. However, regardless of activation, in both cases, PDE3B has been found to hydrolyse cAMP to 5'AMP, decreasing PKA phosphorylation of downstream proteins, such as HSL and perilipin. Therefore, insulin receptor activation and  $\beta$ -adrenergic receptor activation work reciprocally to regulate NEFA and glycerol release. Signals required to activate these receptors can initiate from other tissues, and extracellular control of adipose tissue has been documented in great detail, including sympathetic nervous system (SNS) control of insulin release from pancreatic  $\beta$ -cells (Lustig, 2008), and activation and release of catecholamines, as reviewed by Bartness et al. (2010).

## **Extracellular signalling**

For many years, research on the extracellular regulation of adipocyte metabolism has focused on hormones and other circulating factors, identified for their effects on energy storage and release, including insulin, leptin, adipokines and from the gastrointestinal tract (GI), ghrelin. Within the brain, receptors for ghrelin, leptin and insulin have been identified in the ventromedial hypothalamus (VH). Signalling by these compounds initiating from out with the central nervous system (CNS), ascending to the brain have previously been described as afferent signalling (Lustig, 2008). Briefly, leptin and insulin are secreted in the fed state, and have been found to act by, amongst other things, increasing the melanocortin receptor 4 (MC4R) occupancy within the CNS. Activation of this receptor is associated with increased movement, energy expenditure and therefore increased weight loss.

The complementary descending efferent pathway has been investigated more recently, as the principal initiator of lipolysis has only been described within the last 10 years. In a review by Bartness et al. (2010), data showing signals emanating from the CNS, and more specifically the sympathetic nervous system (SNS), were involved in the inhibition of lipolysis. As complete shutdown of lipolysis through local inhibition was not achievable; another form of innervation was suggested to be controlling white adipose tissue lipolysis in mammals. The SNS was found to act directly on white adipose tissue (WAT) and therefore control energy release from adipocytes to a certain extent. Bamshad et al. (1998) as cited in Bartness et al. (2010) identified the brain regions involved in the initial innervation of WAT through the utilisation of retrograde viral tract tracer pseudorabies virus (PRV). A trace was performed back along the neuron from the central premotor sympathetic neurons revealing the 'CNS-SNS-WAT' circuit. To confirm their hypothesis, the Siberian hamster was used to illustrate that denervation of SNS neurons blocked WAT lipid mobilisation, and conversely increasing melanocortin receptor (a co-localising receptor with SNS outflow neurons) agonism resulted in an increase in phosphorylation of HSL and perilipin. This group also identified that whilst the SNS projections originated in the brain, the neuron terminals within the adipose tissue were not uniform. Youngstrom and Bartness (1995) demonstrated that WAT SNS innervation depended on many factors including internal region of the fat pad (inguinal, epididymal etc.) and food deprivation. This irregularity was also linked to norepinephrine turnover (NETO), showing that high NETO was linked with high levels of adipocyte innervation and *vice versa*, with an overall

influence on the likelihood of lipolysis occurring within adipocytes. The presence of surface receptors, sensitive to such innervation are therefore also important to the activity of adipocyte lipolysis. This is evident in a study showing subcutaneous adipocytes isolated from female plastic surgery patients had a large number of  $\alpha_2$ -AR and the highest % inhibition of lipolysis in the presence of a full  $\alpha_2$ -AR agonist (Castan et al., 1994). Also, the effects of GPCR activation have also been widely documented on adipose tissue lipolysis. (Ge 2008) demonstrated that a decrease in % glycerol release observed in wild type animals in the presence of SCFA propionate and acetate was ablated in GPR43 deficient mice.

### **Adrenergic receptors: The link between extracellular and intracellular control of adipose tissue lipolysis**

There is a complex network of both internal and external pathways involved in TAG breakdown, fatty acid and glycerol release as well as re-esterification. The presence of adrenergic receptors (AR) in the membrane of adipocytes is well documented in the literature (Collins and Surwit, 2001), and these receptors have been found to act as a link between some of the external and internal pathways. Activators, such as catecholamines, increase in abundance after SNS signalling, and are responsible for the activation of AR. There are two main groups of AR,  $\beta(1,2,3)$  and  $\alpha(1,2)$ , and both of these receptors are situated in the cellular membrane and are coupled to a family of GPCR. The GPCR is made up of an  $\alpha$ ,  $\beta$  and  $\gamma$  subunit. The two main  $\alpha$  subunits related to the activation/inhibition of the lipolytic pathway include the stimulatory subunit  $G\alpha_s$  and the inhibitory subunit  $G\alpha_i$ . GPCR linked receptors are expressed in multiple tissues, as HM47/HM47A has been identified in WAT, spleen and lung of humans (Pike, 2005, Tunaru et al., 2003). AR can be more tissue specific, with  $\beta_3$ -receptors more commonly expressed in adipose tissue, compared with  $\beta_1$  and  $\beta_2$  ARs, which are expressed throughout human tissues (Collins and Surwit, 2001).

It has been reported that the ratio of  $G\alpha_i$  to  $G\alpha_s$  may play an important part in regulation of lipolysis in adipose tissue. Factors affecting activation ratio of GPCR have been proposed, including type and location of adipose depot, as well as sympathetic innervation and norepinephrine turnover (NETO) (Bamshad et al., 1998). The downstream signalling associated with binding of catecholamines to the surface of a  $\beta$ -AR, such as  $\beta_3$  on adipose tissue depends on the coupled GPCR. In the case of  $G\alpha_s$  activation, membrane-bound adenylate cyclase would become activated leading to an

increase in cAMP availability and subsequent increase in lipolysis. However, if  $G\alpha_i$  were to be stimulated through a  $\beta_3$  agonist, it would result in an inhibitory effect on adenylate cyclase. Adenylate cyclase acts as a surface protein linking external activation to downstream control of lipolysis. The stimulatory effect exerted on adenylate cyclase by  $G\alpha_s$  increases circulating cAMP and subsequent increases in PKA phosphorylation of downstream proteins and lipases (Fig 1.2).

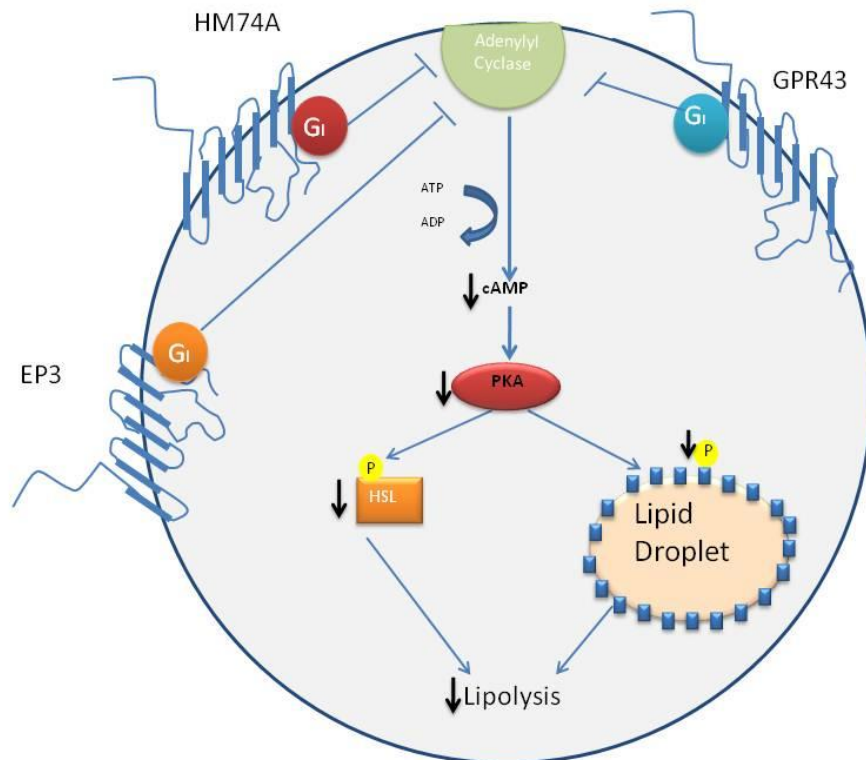


Fig 1.4 The adrenergic G-protein coupled receptor cascade: The involvement of potential inhibitory GPCR that may influence cAMP and the subsequent decrease in lipolysis.  $G_i$ , inhibitory G protein-coupled receptor; cAMP, cyclic adenosine monophosphate; PKA, protein kinase A; HSL, hormone sensitive lipase; P, phosphate; ATP, adenosine triphosphate; ADP, adenosine diphosphate.

The inhibitory effects of  $G\alpha_i$  however, decreases overall cAMP levels and therefore inhibits PKA activity (Wettschureck and Offermanns, 2005), illustrated in Fig 1.4. PKA activation through increased cAMP availability results in the phosphorylation of both HSL and perilipin. Both proteins subsequently aid in the hydrolysis of fatty acids from the glycerol backbone, increasing overall lipolysis in adipocytes, as reviewed by Duncan et al. (2007).



## **GPR43**

GPR43, part of the seven transmembrane receptor super-family has been identified as a receptor for SCFA in adipocytes amongst other tissues (Hong et al., 2005). Ligands with the highest efficacy have been identified as acetate (C2) and propionate (C3) (Le Poul et al., 2003). Important tissue specific differences are present between GPR43 and its subfamily members GPR40 and GPR41. GPR43 expression can be seen in a multitude of different tissues including spleen, colon, stomach and adipose tissue, whereas, GPR41 expression is much more restricted and does not appear to be expressed in adipose tissue at all (Hong et al., 2005). GPR43 has been linked to the  $G\alpha_i$  inhibitory pathway responsible for the inhibition of membrane-associated adenylate cyclase (Le Poul et al., 2003) and subsequent reduction in free fatty acid release (Ge et al., 2008). Another important feature of this GPCR subfamily, is evidence to suggest that SCFA not only inhibit lipolysis, but also increase lipogenesis. Acetate has been found to contribute to an increase in lipogenic activity influencing the re-esterification of fatty acids into triacylglycerides through the activation of GPR43 (Hong et al., 2005). Evidence for GPR43 activation of two pathways may be explained by a complementary mechanism; as it is possible that activation of GPR43 receptors by SCFA may inhibit adipocyte lipolysis, whilst also increasing lipogenesis, both reducing the availability of free fatty acids.

## **HM74A/GPR109A and PUMA-G**

The small human carboxylic acid receptor HM74A/GPR109A and PUMA-G in rodents is found at high concentration in white adipose tissue. In 2003, HM74 was reported by Tunaru et al. (2003) to be responsible for the anti-lipolytic function in white adipose tissue; however, there is now evidence to suggest the close homologue HM74A is responsible for this activity (Zhang et al., 2005). The ligand with the highest affinity for this receptor has been identified as nicotinic acid (NA) and is prescribed regularly in the treatment against cardiovascular disease (CVD). NA has been found to increase preferential high-density lipoproteins (HDL) and reduce levels of low-density lipoproteins (LDL) and very-LDL (VLDL) with an overall reduction in CVD (Pike, 2005). The concentration at which NA is present in the circulation is however, too low to act as the endogenous agonist for HM74A (Tunaru et al., 2003). There is evidence to suggest other possible natural ligands. Due to the similar small carboxylic acid

structure common between niacin and the SCFA acetate and propionate, they too may possess affinity for HM74A.

### **EP3 and Prostaglandin E<sub>2</sub>**

Prostaglandins (PG) are commonly ascribed to the inflammatory response producing effects such as flushing and itching. PGE<sub>2</sub>, derived from arachadonic acid, has been identified at high concentrations surrounding adipose tissue. The prostaglandin receptor EP3 has also been found in adipocytes at roughly ten times the concentration of any other prostaglandin receptor (Jaworski et al., 2009). The activation of certain inhibitory GPCR and the release of prostaglandins surrounding adipose tissue was investigated by Castan et al. (1994). EP3 found on the surface of adipocytes is activated by PGE<sub>2</sub>. Generation of PGE<sub>2</sub> has been linked to the presence of adipose phospholipase A (AdPLA). From reports by Jaworski et al. (2009), it was identified that genetic ablation of the *Adpla* gene in rodent studies resulted in a decrease in WAT mass, supported by evidence of a 2-fold increase in cAMP levels compared to wild type rodents. It was also identified that the *Adpla* null mice had a much lower level of PGE<sub>2</sub> in adipose tissue compared to wild type and therefore decreased EP3 receptor activation. This lead to the postulation that the decrease in Gα<sub>i</sub> coupled EP3 activation, and consequently, the overall increase in cAMP could account for the physiological changes observed in these animals. NEFA and TAG were found to be lower in *Adpla* null mice contrary to expectations. The molar ratio of fatty acids to glycerol release from WAT was also lower in *Adpla* null mice compared to wild type, however, subsequent experiments showed an increase in oxidative metabolism of proteins and an increase in fatty acid oxidation of 37 % compared to wild type mice. The decrease in NEFA and TAG appearance in this study were therefore accounted for by an increase in oxidative metabolism (Jaworski et al., 2009). Furthermore, it was identified by Chang-sheng Chang (1997) that Chinese hamster ovary (CHO) cells expressing EP3 receptors became activated by TEI-3356, containing a negative carboxylic acid terminal, resulting in a decrease in cAMP (Jaworski et al., 2009, Chang-sheng Chang, 1997). Similarities can be drawn between the presence of a small carboxyl group in each agonist of the GPCR Gα<sub>i</sub> mentioned previously, and a reduction in adipocyte lipolysis. Speculatively, activation of SCFA receptors (GPR43) resulting in a reduction of lipolysis, may be compounded if activation of other receptors, such as HM74A and EP3, are susceptible to agonists containing a similar carboxyl tail region. However, it should also be

considered that side effects for patients commonly prescribed non-steroidal anti-inflammatory drugs (NSAID), which reduce the presence of natural prostaglandins do not include weight gain (NHS, 2012).

The regulation of lipolysis is complex, and many studies have contributed to our current understanding of adipose tissue metabolism and function, as it has been discovered in recent years that adipose tissue is much more metabolically active than previously thought. However, there are still gaps in our knowledge. Due to the increased incidence of obesity and related disorders in the UK and worldwide, including type II diabetes, experiments on the molecular mechanism(s) underlying such changes in the metabolism of adipose tissue are of interest, as they may lead to novel therapeutic targets for the treatment of these disorders. The pathway of lipolysis is predominantly controlled by two converging mechanisms, GPCR activation and insulin receptor activation, with the overall outcome of maintaining adipocyte homeostasis. Increases in acetate availability have been found to affect adipose tissue metabolism by reducing lipolysis, as a measure of glycerol and NEFA release, however, at supra-physiological concentrations (above 1mM). Identification of adipocyte surface receptors, such as GPR43, specific for short chain fatty acids, may therefore be of value when considering new therapeutic targets for the side effects of obesity and related disorders. Interest in these receptors has increased as the intracellular mechanism(s) by which SCFA effect lipolysis are still unknown. Receptor activation with physiological concentration of SCFA may contribute to overall adipocyte homeostasis; however, it is of interest to establish if supra-physiological SCFA acetate availability may induce a beneficial response including the reduction of side effects of type II diabetes, including dyslipidaemia, as previous studies have shown, that artificially induced acetate concentrations, as high as 10 mM reduce lipolysis, as a measure of glycerol and NEFA release. Therefore with the use of the *in vitro* 3T3-L1 cell line and *ex vivo* primary adipocyte cells, this thesis aims to identify the intracellular mechanism(s) underlying the effect of supra-physiological concentrations of short chain fatty acid, acetate, on lipolysis, by utilising phosphorylation of the key lipolytic lipase HSL, and comparing this with changes to NEFA, glycerol and TAG concentration.

# **Chapter 2**

## **Methods and Materials**

## **Cell culture of murine 3T3-L1 adipocytes**

### **Culture media**

Murine 3T3-L1 cells were purchased frozen in liquid nitrogen from Zen-Bio, at passage 8. Cells were not expanded past passage 12. All media used for cell culture were acquired from Zen Bio (USA), and therefore the concentration of compounds used in each plating media could not be obtained. Plating medium PM-1-L1 for pre-adipocyte cell culture contained Dulbecco's Modified Eagle Medium (DMEM), high glucose, Hydroxyethyl Piperazineethanesulfonic Acid (HEPES) pH 7.4, bovine calf serum (BCS), penicillin, streptomycin, amphoterecin B. Cell differentiation media DM-2-L1 contained DMEM/Ham's F-12 (1:1, v/v), HEPES pH 7.4, fetal bovine serum (FBS), biotin, pantothenate, human insulin, dexamethasone, penicillin, streptomycin, amphoterecin B, isobutylmethylxanthine (IBMX) and PPAR $\gamma$  agonist. Mature adipocyte medium AM-1-L1-PRF contained, DMEM/Ham's F-12 (1:1, v/v), HEPES pH 7.4, FBS, biotin, pantothenate, human insulin, dexamethasone, penicillin, streptomycin and amphoterecin B. With the exception of bovine serum albumin-fatty acid free (BSA-FFA) (PAA Cell Culture Company) all treatment reagents were purchased from Sigma Aldrich. DMEM-PRF (phenol red free) supplemented with 2 % (w/v) BSA-FFA was the basis of all treatment media. Isoproterenol hydrochloride was reconstituted in dimethyl sulphoxide (DMSO), and sodium acetate was reconstituted in sterile water (H<sub>2</sub>O) before use. Insulin from bovine pancreas was used throughout at a final concentration of 1.34  $\mu$ M. Phosphate buffered saline (PBS) solution (20x) was prepared by dissolving 22.88 g Na<sub>2</sub>HPO<sub>4</sub>; 160.00 g NaCl; 4.00 g KH<sub>2</sub>PO<sub>4</sub> and 4.00 g KCl in 1 litre of sterile H<sub>2</sub>O and adjusting to pH 7.2 with 10 M NaOH. A 1x solution was prepared by diluting 50 ml in 950 ml sterile H<sub>2</sub>O and filtering before use.

### **3T3-L1 cell culture**

Cells were fed according to the feeding manual provided by Zen-Bio (2010), following precise media changes for each well format. Cells were plated in PM-1-L1 at a density of 5000 cells/cm<sup>2</sup>, in either 6 or 12 well plate formats. Every two days, 60 % PM-1-L1 media was aspirated and replaced with 60 % fresh PM-1-L1 media over a period of 5 days until cells were 100 % confluent. Cells were then left for a further 48 hrs to initiate growth arrest, during which time the media was not changed. The cells were then initiated to differentiate by aspirating all PM-1-L1 and replacing with DM-2-L1. The cells were left to incubate for 72 hrs, again during this time the media was not

changed. After differentiation, day 0, 60 % DM-2-L1 was aspirated from each well and replaced with 80 % AM-1-L1-PRF. This occurred every 2 days allowing for a gradual change from DM-2-L1 to AM-1-L1-PRF media. Visualising lipid accumulation using the Oil Red O stain, mature 3T3-L1 cells were deemed sufficiently lipid filled for treatment on day 9 post-differentiation, within the manufacturers' guidelines of 7 - 14 days.

### **3T3-L1 cell treatment**

On day 9 post-differentiation, cells were washed in 1 x PBS before DMEM-PRF media containing 2 % (w/v) BSA-FFA plus a treatment of 1. Basal 2. Sodium acetate (4 mM) 3. Insulin (1.34  $\mu$ M) 4. Sodium acetate + Insulin (NaAc+Ins.) 5. Isoproterenol (Iso) (5  $\mu$ M) 6. Iso+Acetate 7. Iso+Insulin 8. Iso+NaAc+Ins., unless otherwise stated. For sodium acetate treatments, cells were pre-incubated in the presence of 4 mM acetate for 30 minutes prior to PBS cell wash step. Samples of treatment medium were collected every 60 minutes over a maximum period of 180 minute and stored on ice. After the last sample was collected, cells were lysed in a 1 x Lysis buffer (Cell Signalling), sonicated for 10 x 1 second bursts before being centrifuged at 10,000 x g for 10 minutes at 4°C. Treatment media samples were stored at -20°C until ready for glycerol and NEFA analysis and cell lysis supernatants were stored at -80°C until ready for triacylglycerol analysis. Protein concentration was measured in both the pellet and the supernatant fractions collected on the same day as sample collection, using the method described below.

## **Isolation of primary mature adipocytes**

### **Culture media**

All cell culture reagents were purchased from Sigma Aldrich with the exception of fatty acid free bovine serum albumin (BSA-FFA) and bovine serum albumin (BSA) (PAA Cell Culture Company). Krebs Ringers Bicarbonate Hepes Albumin (KRBHA) washing buffer included dissolving 0.5 mM  $\text{CaCl}_2$ , 238 mg/100 ml HEPES, 108 mg/100 ml Glucose and 3.5 % (w/v) BSA in 100 ml Krebs Ringers Bicarbonate (KRB) buffer (Zen Bio KRB-1000). The tissue digestion buffer (DB) included reconstitution of 0.5 mM  $\text{CaCl}_2$ , 238 mg/100 ml HEPES and 108 mg/100 ml glucose in 100 ml KRB with the addition of 1 mg/ml collagenase (Sigma C6885) and 289  $\mu\text{g/ml}$  liberase (ROCHE 5401119001) immediately before use. Mature isolated cells were plated in endothelial cell medium (Sigma C1556) containing 0.5 % BSA-FFA.

### **Mature adipocyte isolation**

All animal work was completed at the Roslin Institute, Edinburgh. An established Home Office licence for animal testing at the Roslin Institute covered the euthanasia process described for this study. All mice were euthanized by spinal dislocation by a trained member of staff with a personal Home Office licence. Fat pad isolation was also undertaken at the Roslin Institute, before isolated cells were transported back to Heriot-Watt University for analysis. No ethical approval was needed for this experiment at either institute. 6 x 16 week old female C57BL6/J mice were purchased from Charles River on the day of treatment. After mice were sacrificed by spinal dislocation, perigonadal/epididymal (PG/ED) fat pads were excised less than 15 minutes later (Fig 2.1). Firstly, each set of fat pads were washed in KRBHA buffer at 37°C and transferred to 5 ml fresh KRBHA. Fat pads were cut into small 2 - 4 mm pieces and placed in 5 ml tissue DB for 45 minutes with continuous shaking at 37°C. Isolated mature adipocytes were filtered through a 225  $\mu\text{m}$  nylon mesh filter syringe into a sterile 15 ml falcon tube and centrifuged for 10 min at 1100 rpm at room temperature. It is unlikely at this stage that only adipocytes were isolated during the washing and centrifugation steps, and the potential for other cells including macrophages to be present should be noted. Isolated cells were pooled from 6 mice and split between one full panel of treatments (basal, acetate, insulin, NaAc+Ins., isoproterenol, Iso+Acetate, Iso+Insulin and Iso+NaAc+Ins.) and washed a further 2 times in KRBHA for 1 minute at 500 rpm. After the final wash step, 150  $\mu\text{l}$  packed cells were plated in 600  $\mu\text{l}$  plating

media in a 12 well plate format. Cells were then placed at 37°C for 4 hrs to stabilize before the addition of the experimental treatments. All experiments were run in duplicate.

### **Primary mature adipocyte cell treatment**

After isolation and recovery, PG/ED adipocyte cells were subsequently treated with sodium acetate. The plating media of these cells was spiked with 4 mM sodium acetate exactly 30 minutes prior to the start of the treatment protocol. At time 0, each well was spiked with a combination of stock isoproterenol (500  $\mu$ M), acetate (400 mM) and insulin (134  $\mu$ M) to reach a final concentration of 5  $\mu$ M isoproterenol, 4 mM sodium acetate and 1.34  $\mu$ M insulin. Samples of treatment media were collected every 60 minutes over a maximum period of 180 minutes and stored on ice. After the last sample was collected, cells were lysed in a 1 x Lysis buffer (Cell Signalling), sonicated for 10 x 1 second bursts and centrifuged at 10,000 x g for 10 minutes at 4°C. Treatment media samples were stored at -20°C until ready for glycerol and NEFA analysis. Protein concentration was measured on the supernatant fraction collected on the same day as sample collection, using the method described below.



Fig 2.1 Perigonadal/Epididymal fat pads (arrows) isolated from a 16 week old female C57BL6 mouse.



## **Measurement of total protein**

Total protein content for each well was measured in order to allow normalization between samples, as mature adipocytes were unable to be counted by haemocytometer. Adipose cells are extremely sensitive and cannot have direct contact with glass, as the surface tension ruptures the cell wall. The Bradford method was chosen as it is a one-step method, sensitive and accurate with most reagents, however a noted disadvantage is a non-linear correlation. This is overcome by generating a new calibration curve every time. By combining the results from both pellet and supernatant fractions we were able to account for both membrane bound proteins and soluble intracellular proteins.

### **Bradford reagent**

Bradford reagent was prepared by dissolving 50 mg Coomassie Brilliant Blue (Sigma) in 25 ml 95 % ethanol, adding 50 ml 85 % (w/v) phosphoric acid and diluting to a final volume of 500 ml. Reagent was filtered before use.

### **Bovine serum albumin (BSA) standard**

Total protein standards were prepared by dissolving BSA (PAA Cell Culture Company) in warmed sterile water at a concentration of 1000 µg/ml. A set of serial dilutions were prepared fresh from the standard everyday including 1000, 500, 250, 125, 63, 31 and 0 µg/ml. All standards were assayed in triplicate.

### **Bradford reagent protein assay**

Prior to the determination of total protein, cells were lysed (Cell Signalling Technology) on ice for 5 minutes, sonicated for 10 x 1 second bursts and centrifuged at 10 000 x g for 10 minutes at 4°C. The supernatant was aspirated to a fresh tube and the pellet was re-suspended in 100 µl sterile water. Each pellet and supernatant sample was diluted 1 in 10 with sterile water before 10 µl was pipetted in triplicate into a flat bottomed 96-well plate. Standards were also added to the plate in triplicate. Bradford reagent, 200 µl, was added into each well and the plate was read immediately on a SpectraMax M5 platereader at wavelength 595 nm. Concentration of total protein in µg/ml was calculated using the re-arranged linear equation  $X = (Y-C)/M$ , generated from the standard curve (Bradford, 1976).

### **Alamar blue (Resazurin) cell cytotoxicity assay**

A 100 x stock solution of resazurin (Sigma) was acquired by reconstituting 1 mg/ml in 1 x PBS. The working concentration was achieved by diluting the 100 x stock solution in cell culture media and warming to 37°C before use. The positive control was achieved by making a 1 % (v/v) solution of Triton X-100 in 1 x PBS.

### **Cell cytotoxicity assay**

Firstly, the positive control was achieved by the addition of 1 % Triton X-100 solution to one well and leaving it to incubate for 1 hr prior to incubation with 1 x resazurin. At least one well was left untreated and kept in original AM-1-L1-PRF media throughout the duration of the experiment, to be used as the negative control for this assay. After the incubation of the positive control was complete, all cell media was aspirated from each well and 0.1 ml/cm<sup>2</sup> of 1 x resazurin solution was added, followed by a 4, 6 and 20 hr incubation at 37°C. .

## **Lipid staining with Oil Red O**

A stock solution of Oil Red O was prepared by dissolving 0.7 g Oil Red O in 200 ml isopropyl alcohol (2-propan-1-ol). Bakers Formalin was prepared by adding 10 ml 37 % formaldehyde and 10 ml of 10 % (w/v) calcium chloride aqueous solution to 80 ml of sterile water before being mixed and stored at 4°C. Glycerol gelatine was prepared by adding 5 g gelatin and 50 ml glycerol to 50 ml sterile water before being mixed gently at 50°C. Glycerol gelatin was also stored at 4°C, but was heated to 55 - 60°C before use. Harris Hematoxylin (Sigma) and Hanks balanced salt solution (HBSS) were stored at 4°C before use.

### **Oil Red O stain**

Firstly a 60 % Oil Red O solution was prepared by diluting stock Oil Red O with sterile water at a ratio of 3:2, and filtering through a Whatman filter paper. Harris hematoxylin was also filtered before use. Treatment media was removed from each well and cells were washed 2 - 3 times in HBSS, blotting any extra buffer with a dry paper towel. Each well was fixed with 0.2 ml/cm<sup>2</sup> Bakers Formalin at 4°C for 30 minutes, after which excess fixative was poured off and blotted with dry paper towel. This was followed by the addition of 0.2 ml/cm<sup>2</sup> 60 % Oil Red O to each well and was left to incubate for 10 minutes at room temperature. Excess stain was poured off and wells were rinsed under continuous water until the water ran clear. Excess water was blotted with paper towel before the cells were counterstained with Harris Hematoxylin by adding 0.2 ml/cm<sup>2</sup> per well and left to incubate for 2 minutes at room temperature. Excess hematoxylin was then poured off and the cells were rinsed under running water for 5 minutes. Excess water was blotted with paper towel and 2 - 3 drops of warmed glycerol gelatin was added to each well and topped with a cover slip (Hausman et al., 2008). Cells were stained with Oil Red O and fixed on 4 separate days to visualize TAG accumulation.

## **Transfection of GFP into mature 3T3-L1 cells**

### **Reagents**

The treatment media was made using DMEM-PRF, high glucose supplemented with 1 % antibiotics (Penicillin/Streptomycin). Stock aliquots of green fluorescent protein (GFP) were kept at a concentration of 1 µg/µl and stock Lipofectamine (Invitrogen, UK) at a concentration of 1 mg/ml. A weight of 20 g Mowoil was resuspended in 80 ml of PBS pH 7.4, before 40 ml of water free glycerol was added. This was then left to stir over night at room temperature. Aliquots were then poured and centrifuged at 2500 rpm for 30 min. The supernatant was then removed and frozen at -20°C until use.

### **GFP transfection**

For transfection of GFP into 3T3-L1 cells, the cells were initially plated on cover-slips in a 24 well plate format. Pre-adipocytes were grown to confluence and differentiated as described on page 16. Firstly, 4 µl GFP (4 µg) was added to an aliquot of 250 µl treatment media in a single micro-tube. In another micro-tube containing 250 µl treatment media, 10 µl of Lipofectamine was added and mixed gently. Both tubes were then left to incubate for 10 min at room temperature. At the end of the incubation 1 tube of DNA/media was mixed with 1 tube of Lipofectamine/media, and left to incubate for a further 30 min at room temperature. Medium covering the cells was removed and the GFP/Lipofectamine/media mix was added to the cells (~500 µl/well). Cells were left to incubate for a further 48 hrs at 37°C, 5 % CO<sub>2</sub>.

Finally, after 48 hrs incubation, the cells were fixed by initially removing the medium from the well followed by washing the cell 3 x with 1 ml PBS. A small volume of 3 % formaldehyde was then added to the cells and left to incubate for 30 min at room temperature. Coverslips were then washed a further 3 x with PBS before being inverted and mounted onto glass slides using Mowoil.

## Measurement of NEFA into culture media

NEFA accumulation in cell culture media was measured using the following reagents, NEFA HR II R1 (434-91795); NEFA HR II R2 (436-91995) and NEFA HR Standard (270-77000), and analysed on a SpectraMax M5 plate reader (Amersham Bioscience) at 550 nm. Each reagent was provided by WAKO and used according to the manufacturer instructions.

### NEFA buffers (R1 and R2)

Buffers R1a and R2a were reconstituted with R1 and R2 respectively. All reagents were mixed thoroughly before use. The 1 mmol/L standard solution was used for a 1 point calibration. Sterile water was used as the blank and this absorbance reading was subtracted from all samples and standard absorbance readings.

### NEFA assay

Briefly, to each well of a flat bottomed 96-well plate, 4 µl of sample/standard/blank was added in triplicate followed by the addition of 160 µl of reconstituted R1a. The plate was shaken and placed at 37°C 5 % CO<sub>2</sub> for 10 minutes exactly. The plate was removed from the incubator and 80 µl of R2a was pipetted into each well and mixed thoroughly, before being incubated again at 37°C, 5 % CO<sub>2</sub> for 10 minutes exactly. Finally the plate was read at 550 nm and all samples were analysed in triplicate. Concentration of NEFA in mmol/L was measured at absorbance 550 nm and calculated according to the following equation:

$$(1) \text{ NEFA-HR(2) concentration (mmol/L)} = \frac{\text{Abs (Sample-Blank)}}{\text{Abs (Standard-Blank)}} \times \text{Standard conc. mmol/L}$$

## Measurement of glycerol into culture media

The measurement of glycerol in the cell culture medium was adapted from the original paper by Boobis and Maughan (1983).

### Potassium phosphate buffer

The basis of the potassium phosphate buffer required two separate preparations of potassium phosphate:

1. 10 mM potassium phosphate dibasic ( $K_2HPO_4$ )
2. 10 mM potassium phosphate monobasic ( $KH_2PO_4$ )

A volume of 25.02 ml 10mM  $K_2HPO_4$  was added to 4.98 ml 10mM  $KH_2PO_4$ , before 20 ml of the combined buffer was diluted in 60 ml sterile water. Following that, 42.28 g of ammonium sulphate (3.2M) was added and the buffer corrected to pH 7.5. Finally 200 mg BSA was added and stirred gently until completely dissolved. The final concentration of 2 mM potassium phosphate buffer containing 3.2 M ammonium sulphate 2 % BSA (w/v) pH 7.5 was achieved by diluting the buffer to a final volume of 100 ml with sterile water.

### Glycerol buffer

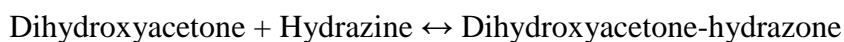
Firstly, 2 ml hydrazine hydrate was added to 10 ml of 2-amino-2-methylpropanol, before 29.4 mg ethylenediaminetetraacetic acid (EDTA) was added. Sterile water was then added to the buffer before being adjusted to pH 9.9 with HCL and diluting to a final volume of 100 ml. The final concentrations were as follows: 2-amino-2-methylpropanol (0.1 mol/L), hydrazine hydrate (0.2 mol/L) and EDTA (1 mmol/L) pH 9.9.

### Glycerol assay

For the measurement of glycerol, the stoichiometric relationship between glycerol and NADH has been utilized. The assay is based on the following reaction:



The presence of extra  $\text{NAD}^+$  allows the reaction to proceed to the right. Extra hydrazine is present to bring the reaction to completion:



Prior to the determination of glycerol, glycerol dehydrogenase (GDH) was reconstituted at a concentration of 0.5 U/μl in 2 mM Potassium Phosphate Buffer. The co-factor NAD<sup>+</sup> was also reconstituted at 1 mg/30 μl sterile water (50 mM). Firstly, spectrophotometric determination of a single standard was conducted using a 2 mmol/L stock solution. In a 1 ml cuvette 20 μl of GDH and 40 μl NAD<sup>+</sup> were added to 1 ml of glycerol buffer and mixed thoroughly. The cuvette was read at 340 nm before 20 μl of the 2 mM glycerol standard was added and the contents mixed again. The cuvette was left to incubate for 2.5 hrs in the dark at room temperature before being read at 340 nm. The concentration of glycerol in the standard was calculated according to the following equations:

$$\begin{aligned}
 (2) \quad \text{Conc. of NADH (M)} &= \left( \frac{\text{Post Abs.} - \text{Pre Abs.}}{\text{Molar ext co-efficient of NADH}} \right) \\
 \text{Moles per Litre (M/L)} &= \text{M}/1000 \\
 \text{Concentration (mmol/L)} &= \frac{10^6 \times \text{M/L concentration}}{\mu\text{l of sample/standard}} \\
 &= \text{mmol/L}
 \end{aligned}$$

Once the precise concentration of the stock standard had been determined, a serial dilution was made and used for the subsequent calculation of the glycerol in the samples. For the determination of glycerol in each sample, sufficient reaction mixture was made fresh each day. The reaction mixture consisted of 10 μl GDH and 20 μl NAD<sup>+</sup> being added to 1 ml Glycerol Buffer before being mixed thoroughly. To 10 μl of sample, blank and standards in a 96-well plate, 40 μl of reaction mixture was added to each well, mixed thoroughly and left to incubate in the dark at room temperature for 4hrs and 30 minutes, double the time used for the 2 mM standard to reach plateau. After incubation, 200 μl of sterile H<sub>2</sub>O was added to each well and the contents thoroughly mixed. Fluorescence was then measured at excitation 340 nm and emission 460 nm and glycerol concentration of samples calculated from the standard curve using the rearranged linear equation  $X = (Y-C)/M$  and corrected for dilution (Boobis and Maughan, 1983).

## **Trapezoid calculation for total NEFA and glycerol in culture media**

### **Calculating the area under the curve**

The following trapezium rule, as described by Matthews et al. (1990), was used to calculate the total concentration of NEFA and glycerol from 3T3-L1 cells in the presence of treatment conditions over time. If we have a NEFA/glycerol concentration of  $y_1$  and  $y_2$  occurring at time points,  $t_1$  and  $t_2$ , the area under the curve (AUC) is the product of the two time differences and the average of the two measurements. To calculate total accumulation over 180 min at 60 min intervals, the AUC of 0 - 60, 60 - 120 and 120 -180 min are added together:

$$(3) \text{ Trapezoidal Rule} = (y_1 + y_2) / 2 * (t_2 - t_1)$$



## **Measurement of intracellular triacylglycerol**

For the measurement of intracellular triacylglycerol all reagents and chemicals were supplied by Cayman Chemicals. The Triglyceride Colorimetric Assay kit (Cambridge Bioscience CAY10010303) was used to calculate triglyceride in mg/dl according to the manufacturer instructions.

### **Standard diluent assay reagent**

For the final buffer 4ml of the salt solution (5x) was diluted with 16 ml sterile water before using to re-suspend adherent cells and preparing the triglyceride standards.

### **Sodium phosphate assay buffer**

For the final buffer 4ml of the 250 mM sodium phosphate solution (5x) was diluted with 16 ml sterile water for use when preparing the triglyceride enzyme solution.

### **Triglyceride enzyme mixture**

The enzyme mixture was prepared by firstly reconstituting the contents of the vial in 1 ml sterile water in a 15 ml falcon tube wrapped in tin foil. To this, 14 ml of diluted sodium phosphate buffer was added and mixed by inversion.

### **Standard preparation**

From the standard supplied with the kit an initial dilution of 1:5 was prepared by adding 100  $\mu$ l triglyceride standard (1000 mg/dl) to 400  $\mu$ l standard diluent. From the resulting 200 mg/dl standard solution a serial dilution was performed to achieve a final standard curve including 200, 100, 50, 25, 12.5, 6.25, 3.125 and 0 mg/dl.

### **Triglyceride assay**

Prior to the start of the assay cells were washed in ice cold 1 x PBS. To each well, 104  $\mu$ l/  $\text{cm}^2$  of ice cold standard diluent was added. Cells were scraped into suspension with a rubber cell scraper before the cell suspension was sonicated 20 times in one second bursts. The cell suspension was centrifuged at 10,000 x g for 10 minutes at 4°C, before the supernatant was removed and stored on ice. The eight standards mentioned above were prepared and 10  $\mu$ l of each was pipetted in duplicate into the 96 well plate provided. As cell lysis released all stored triacylglycerol into the cell suspension it was important to warm the samples to 37°C before pipetting any sample into the plate, as

centrifugation at 4°C to remove the cell membrane fraction also solidified the lipids. Once the lipids were back in suspension, 10 µl of sample was pipetted in duplicate. To initiate the assay, 150 µl of diluted triglyceride enzyme mixture was added to each well. The plate was then shaken for a few seconds, covered with the plate cover and incubated at room temperature for 15 minutes. The plate was then read at 550 nm and total triglyceride content was calculated according to the following equation, corrected for dilutions and normalised to total protein:

$$(4) \quad \text{Triglyceride (mg/dl)} = \frac{(\text{Corrected absorbance}) - (\text{y-intercept})}{\text{Slope}}$$

## **Western blotting**

### **Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)**

#### **Running buffer**

A 10 x stock solution of running buffer was made by dissolving 30.3 g Trizma base, 144 g glycine and 10 g SDS in 1 L sterile water. The final 1 x running buffer consisted of 100 ml 10 x running buffer diluted in 900 ml sterile water to obtain a final concentration of 25 mM trizma base, 192 mM Glycine and 0.1 % (w/v) SDS.

#### **Lysis buffer**

A 10 x lysis buffer was obtained from Cell Signalling (Cat. 9803). Sterile water was used to dilute the 10 x lysis buffer to a 1 x solution and cooled on ice before use. Protease inhibitors such as phenylmethanesulfonyl fluoride (PMSF) (Sigma P2676) were added at a final concentration of 1 mM immediately before use.

#### **Sample buffer**

A 2 x Tris-Glycine SDS sample buffer was obtained from Invitrogen (Cat.LC2676). Sample buffer was added directly to samples at a ratio of 1:2.

#### **Preparation of lysates**

Firstly, cells were washed in ice cold 1 x PBS. To each well, 200 µl 1 x Lysis buffer was added and left to incubate on ice for 5 minutes. Cells were then scraped into buffer and centrifuged at 10,000 x g for 10 minutes at 4°C. Due to the release of triacylglycerol from the intracellular lipid droplets, the middle supernatant layer was aspirated carefully and centrifuged under the same conditions for a second time.

#### **Electrophoresis**

Samples were mixed with 2 x sample buffer at a ratio of 1:2 before boiling at 100°C for 5 minutes. Samples were flash cooled for 5 minutes on ice before loading into 9 - 15 % SDS PAGE mini-PROTEAN TGX™ pre-cast gels. Electrophoresis was carried out in a mini-PROTEAN Tetra System at 200 V for 40 minutes, or until the bromophenol blue dye reached the bottom of the gel.

## **Protein transfer**

### **Transfer buffer**

A 10 x stock solution of transfer buffer was made by dissolving 30.3 g Trizma Base and 144 g glycine in 1 L sterile water. The final 1 x transfer buffer was achieved by diluting 100 ml 10 x transfer buffer in 900 ml sterile water to obtain a final concentration of 25 mM Trizma Base and 192 mM glycine.

### **Washing buffer – Tris buffered saline with Tween 20 (TBS-T)**

To achieve the final 1 x TBS-T, two buffers were needed. A stock solution of 0.5 M Trizma Base pH 7.5 was made by dissolving 30.3 g Trizma base in 500 ml sterile water, HCL<sup>-</sup> was used to pH to 7.5. The stock solution of 5 M NaCl was made by dissolving 146.1 g NaCl in 500 ml sterile water. Firstly a 1 x TBS concentration of 20 mM Trizma Base and 150 mM NaCl was achieved by diluting 40ml 0.5 M Trizma Base (pH 7.5) and 30 ml 5 M NaCl in 930 ml sterile water. To 999 ml of 1 x TBS, 1 ml Tween 20 was added and mixed thoroughly. TBS-T was chilled to 4°C before use.

### **Blocking buffer**

Non-fat dried milk powder (Marvel) was used as a blocking agent at a concentration of 5 % (w/v) in TBS-T. This was prepared fresh on the day of use.

### **Detection reagents**

LumiGLO detection reagent was purchased from Cell Signalling (#7003) and prepared by the addition of two reagents. Immediately before use, 0.5 ml 20 X LumiGLO and 0.5 ml 20 X Peroxide were diluted in 9 ml dH<sub>2</sub>O and mixed by inversion.

### **Protein transfer**

Proteins separated by SDS-PAGE were transferred to polyvinylidene fluoride (PVDF) membrane prior to protein detection using specific antibodies. Sponges used for transfer were equilibrated in buffer for 20 minutes prior to the start of transfer. PVDF membrane was activated by soaking in 100 % methanol for 1 minute and subsequently washed in distilled water for 5 minutes before being equilibrated in transfer buffer for 20 minutes. Filter pads were soaked in transfer buffer for 1 minute prior to transfer. Before transfer, gels were briefly washed in distilled water. The proteins were

transferred using the Bio-Rad mini-PROTEAN Tetra System for 40 minutes at 100 V in a cooled unit using ice packs and stirrer.

### **Antibody detection**

Phosphorylated hormone sensitive lipase (SER563) was visualised on PVDF membranes using the pHSL<sub>(SER563)</sub> primary antibody from Cell Signalling (#4139). After protein transfer to PVDF, the membranes were washed 1 x 5 min in TBS-T before blocking over night at 4°C on a shaking platform. To ensure equal amounts of protein were added to each lane the membranes were also probed for GAPDH (14C10) as a loading control (Cell Signalling #2118). The following day membranes were washed 2 x 2 min in TBS-T before cutting the membrane at a point between the protein of interest and loading control. Primary antibodies for pHSL<sub>(SER563)</sub> (1:1000) and GAPDH (14C10) (1:1000) were prepared in TBST-T 5 % milk powder and membranes incubated for 1 hr on a rocking platform at room temperature. Membranes were then washed again 6 x for 5 min in TBS-T before the addition of the secondary antibody anti-rabbit IgG HRP linked (1:3000) in TBS-T 2 % milk powder were incubated for 1 hr on a rocking platform at room temperature. Membranes were washed for a final time 6 x 5 min in TBS-T before detection reagents were added. LumiGLO reagents were kept on ice and prepared immediately before use. To each membrane, 10 ml of 1 x detection reagent was added and incubated for 1 minute before blotting excess reagent and placing membranes between two clear acetate sheets. Membranes were visualized on a Bio-Rad ChemiDoc XRS+ Imager using Image Lab 2.0 software for analysis.

# **Chapter 3**

## **Quality Control of Experimental Methods**

## Introduction

The 3T3-L1 cell line has been utilised and optimised as an experimental model since the mid-1970s (Green and Kehinde, 1976, Green and Kehinde, 1975, Green and Meuth, 1974) cited by Lafontan (2012), and was first isolated from Swiss 3T3 mouse embryos. Since then, the 3T3-L1 cell line is now commonly used to study the metabolic function of mature adipocytes (Berggreen et al., 2009, Ge et al., 2008, Mulder et al., 2005, Lasa et al., 2012).

Within the literature, the use of a stimulator of lipolysis is common when studying surface receptor activation and metabolic function of adipose tissue, either *in vivo* or *in vitro* (Mulder et al., 2005, Tavernier et al., 2005, Youngstrom and Bartness, 1995, Koppo et al., 2012, Collins and Surwit, 2001, Collins et al., 2004). Adipocyte stimulation can be induced through activation of surface receptors by endogenous catecholamines, however, the synthetic alternative isoproterenol is also commonly used (Daval et al., 2005, Hong et al., 2005, Yin et al., 2003). The concentration of isoproterenol used to induce adipocyte lipolysis varies within the literature, as evidenced by Hong et al. (2005) utilising 100 nM and Daval et al. (2005) and Yin et al. (2003) utilising 1  $\mu$ M and 10  $\mu$ M, respectively. *In vitro* activation of the lipolytic pathway results in an accumulation of NEFA and glycerol into cell culture media. Studies have utilised the release of these by-products as a measure of change in lipolysis for many years (Nilsson and Belfrage, 1978, Björntorp, 1966). The assays used to measure levels of NEFA and glycerol are extremely sensitive, with a capacity to measure nmole changes in concentration. More recently, changes in protein levels and second messenger concentrations, can be assessed, including HSL and cAMP (Daval et al., 2005, Zhou et al., 2011).

The use of *ex vivo* preparations of mature adipocytes are also used to study the effects of treatments on adipocyte metabolism, including lipolysis. Both mature adipocytes and stromal vascular cell (SVC) fractions can be utilised after excision of the fat pad of interest, as reviewed by Lafontan (2012). The SVC fraction can be filtered to isolate the pre-adipocyte cell fraction and plated before the process of differentiation can occur. The isolation of mature primary adipocytes is also complex and requires a number of techniques and reagents in order to achieve a high yield of cells and maintain cell viability (Rodbell, 1964, Hausman et al., 2008, Ruan et al., 2003).

NEFA, glycerol and TAG assays were chosen as an initial measure of changes in lipolysis, in the presence of the selected treatment compounds isoproterenol, insulin and acetate. Furthermore, as there was no indication within the NEFA assay manufacturer's instructions regarding potential interference of isoproterenol, acetate or insulin, it was hypothesised that isoproterenol, acetate and insulin, either individually or in combination would not significantly interfere with the absorbance reading of the sample. Due to a lack of information in the literature regarding the concentration required to half maximally stimulate ( $EC_{50}$ ) NEFA and/or glycerol release from 3T3-L1 cells with the  $\beta$ -AR stimulator isoproterenol, it was hypothesised that 5  $\mu$ M isoproterenol would produce half maximal stimulation of lipolysis, and that under stimulated conditions, 2 mM sodium acetate would significantly reduce NEFA and glycerol accumulation in the media. It was also hypothesised that the expression of phosphorylated HSL (pHSL<sub>SER563</sub>) in 3T3-L1 cells would increase in isoproterenol stimulated cells compared with the basal state, but that expression of GAPDH would remain unaffected. Finally, it was hypothesised that mature primary adipocytes from 16 week female C57BL6 mice would be most viable after isolation in a combination of both collagenase and liberase.

### **Statistical analysis**

All statistical analysis was performed using IBM SPSS Statistics 20 software. All data were analysed for outliers using boxplots before testing for normality. Data with <50 samples were tested for normality using the Shapiro-Wilk test and data with >50 samples were checked for normality using the Kolmogorov-Smirnov test. Normally distributed data that included both treatment and time parameters were analysed using a 2-Way analysis of variance (ANOVA) whereas data that included only a treatment parameter were analysed using a 1-Way ANOVA. Both tests included a post-hoc Tukey test. For non-parametric data analysis, all samples were deemed independent of each other, and so a Kruskal-Wallis test was conducted followed by pair-wise Mann-Whitney analysis. The mean and standard error of the mean (SE) were used to graph data found to be normally distributed whereas median and quartiles were used for data found to be non-parametric. A  $P$  value of  $\leq 0.05$  was considered statistically significant.



## Results

### 3T3-L1 cell lipid deposition

Over a period of 8 days post-differentiation, it was clear there was an increase in accumulation of triacylglycerol as shown by Oil Red O staining in Fig 3.1 A, B, C and D. In the early stage of differentiation (Fig 3.1 A) only a few cells contained lipid droplets and many still exhibited the long projections from the cell body as seen in pre-adipocytes. After 3 days post-differentiation (Fig 3.1, B), cells were starting to store TAG in multiple small lipid droplets. The majority of these cells had lost their fibroblast-like spiny projections and were forming the recognisable spherical shape of mature adipocytes. By day 5 post-differentiation (Fig 3.1, C), the majority of mature adipocytes contained lipid droplets, however the distribution of lipid deposition was not equal between fat cells. At day 8 post-differentiation (Fig 3.1, D), most mature adipocytes contained lipid droplets and were sufficiently lipid filled to be used in subsequent experiments.

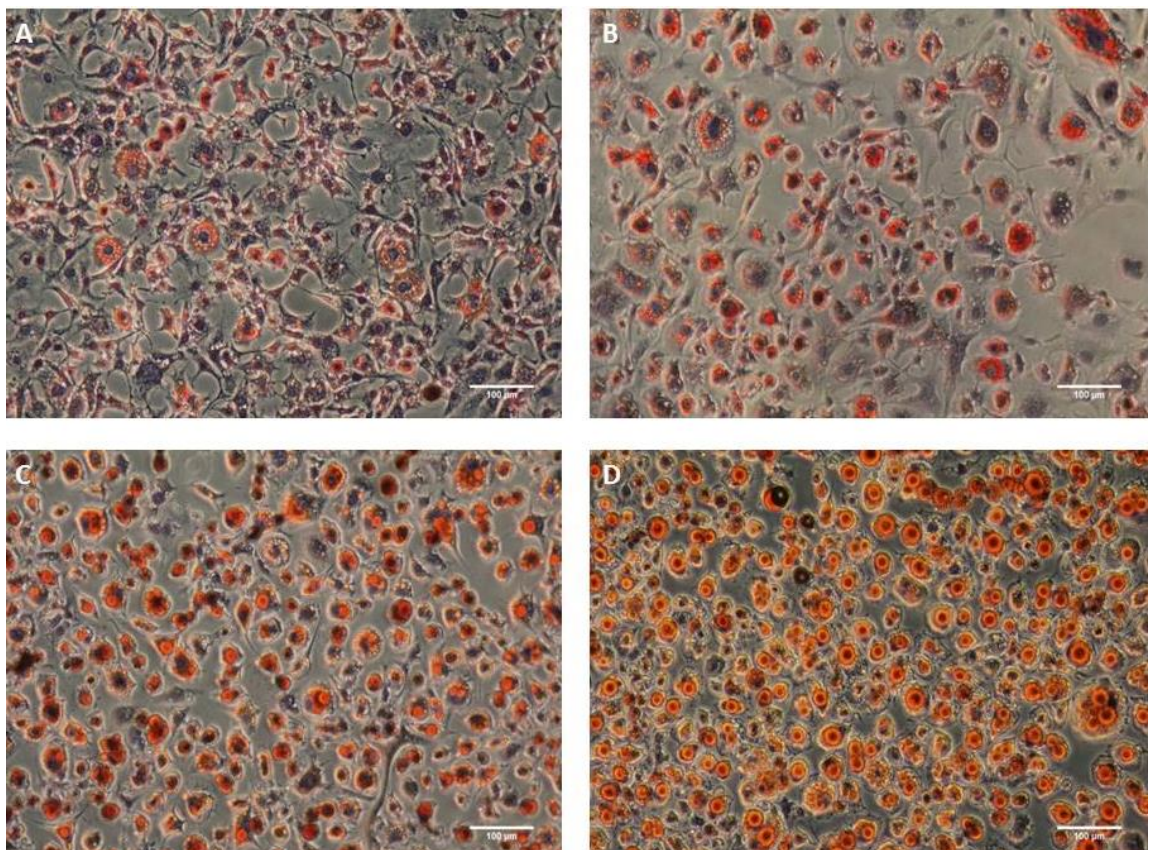


Fig. 3.1 Differentiated mature 3T3-L1 cells stained with Oil Red O. A. Day 1 post-differentiation B. Day 3 post-differentiation C. Day 5 post-differentiation D. Day 8 post-differentiation. Pictures were taken on a Zeiss Axiovert 40C inverted light microscope under 10X magnification.

Lipid droplets also appeared fuller than those in day 5 post-differentiation. In order to confirm sufficient lipid accumulation throughout the whole cell, which could not be achieved using standard microscopy techniques, transfection with green fluorescent protein (GFP) was performed.

From the transfection of GFP into day 9 post-differentiated 3T3-L1 cells, it was possible to visualise multiple lipid droplet formations of varying size through a cross section of the cell (see Fig. 3.2), with the nucleus present at the edge of the cell wall (arrow). In order to identify if the lipid droplets varied in size throughout the whole adipocyte, a visual stack of images was acquired on a Leica SP5 SMD confocal laser scanning microscope. Sections were taken every 0.258  $\mu\text{m}$ , and as shown in Fig. 3.3, lipid droplets appear to vary in size throughout the whole cell. In contrast to Fig 3.2, the nucleus of cell imaged in Fig. 3.3 appears to be in the centre of the cell.

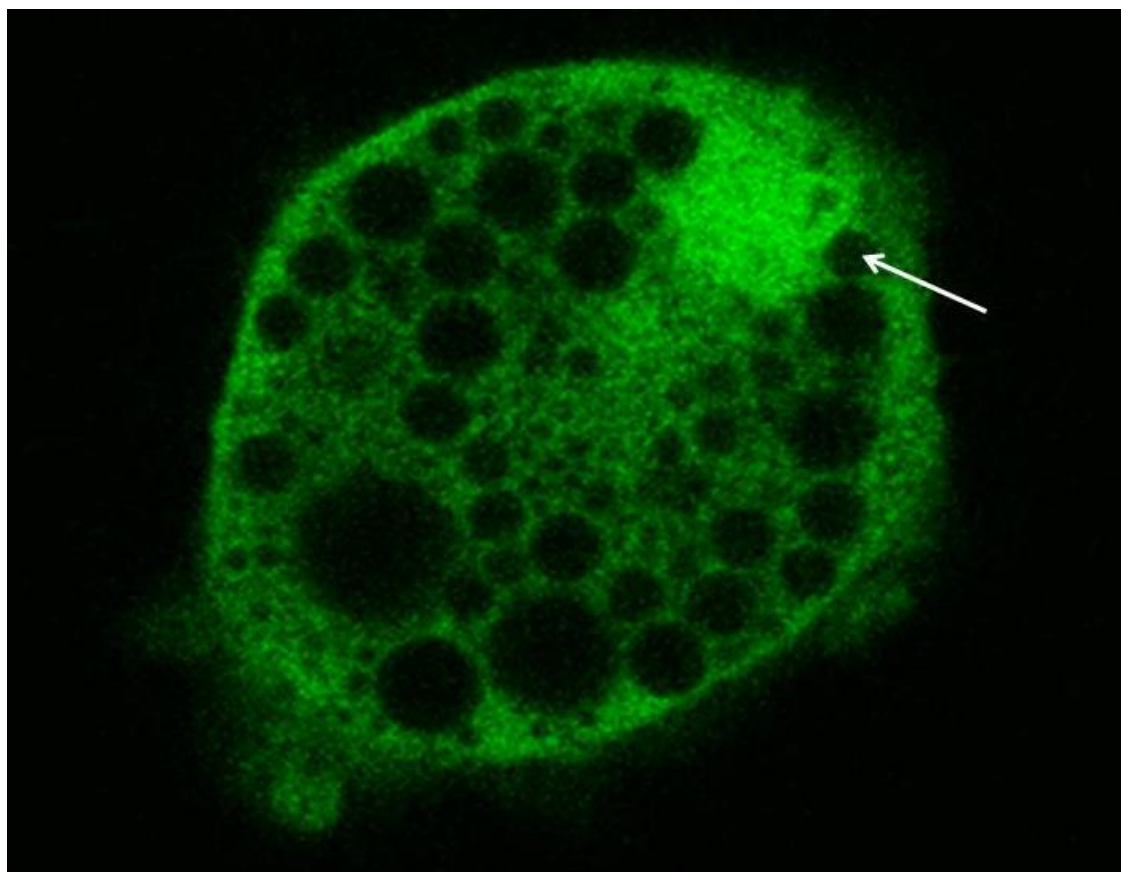


Fig. 3.2 Day 9 post-differentiated 3T3-L1 cell transfected with 4  $\mu\text{g}$  GFP for 48 hrs. Arrow indicates cell nucleus.

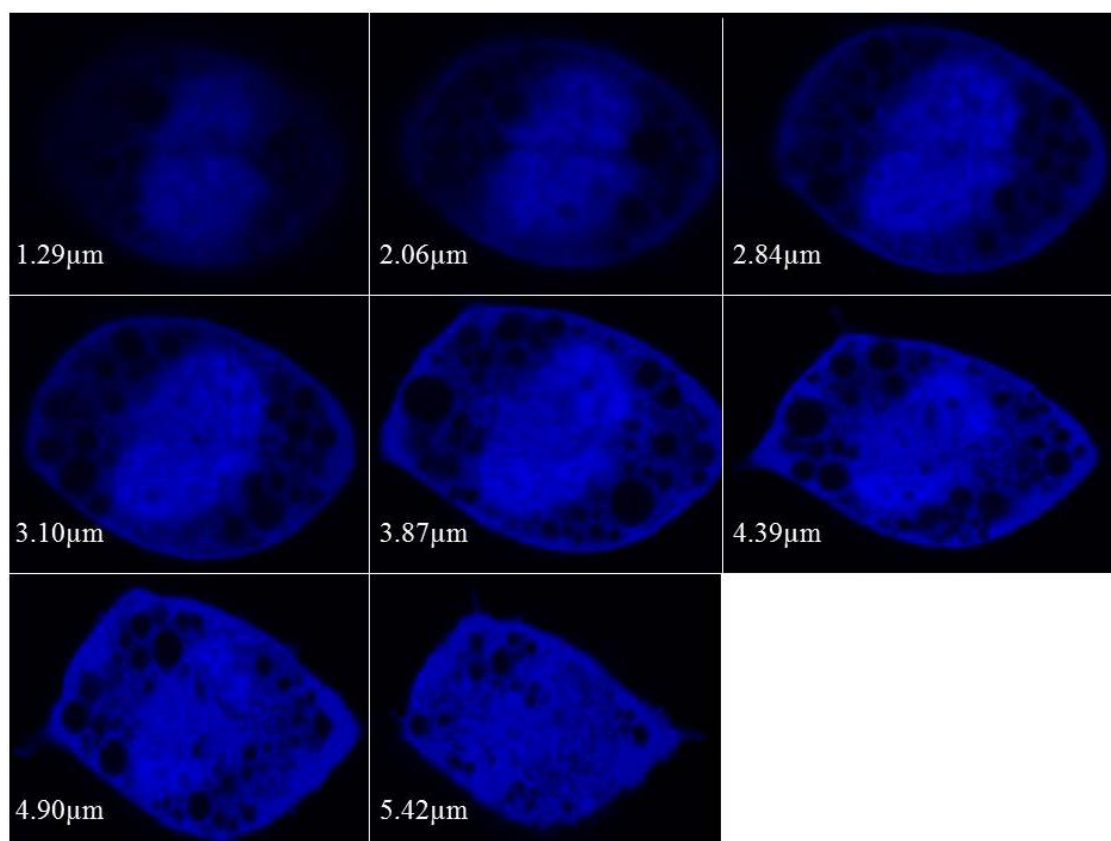


Fig. 3.3 Day 9 post-differentiation 3T3-L1 cells transfected with 4 µg GFP for 48 hrs. Stack images through the cell in 0.258 µm sections.

### Isoproterenol titration on NEFA and glycerol (3T3-L1 cells)

From Fig. 3.4, it was clear that NEFA increased significantly in the presence of each dose of isoproterenol compared with 0 µM after a 120 min incubation (6.25 µM  $P = 0.050$ ; 12.5 µM  $P = 0.050$ ; 25 µM  $P = 0.050$ ; 50 µM  $P = 0.050$  and 100 µM  $P = 0.050$  vs. 0 µM). However, there was no significant difference in NEFA between 6.25 µM and 100 µM isoproterenol (6.25 µM vs. 12.5 µM  $P = 0.127$ ; 12.5 µM vs. 25 µM  $P = 0.827$ ; 25 µM vs. 50 µM  $P = 0.513$  and 50 µM vs. 100 µM  $P = 0.127$ ). In Fig. 3.4, incubation with 12.5 µM isoproterenol resulted in an accumulation of 1.79 µmol/mg protein of NEFA, after 120 min, compared with 20.87 µmol/mg protein in the presence of 10 µM isoproterenol in Fig. 3.6, a 15 fold reduction in NEFA. However, this change is possibly due to a difference in the total protein calculated from each sample. There is evidence to suggest the supernatant fraction of cell lysates can contain  $\geq$  protein than in the insoluble pellet fraction. A lack of protein data from the supernatant fraction of samples used in Fig. 3.6 may have artificially increased the level of NEFA calculated in the presence of 10 µM isoproterenol in Fig. 3.4.

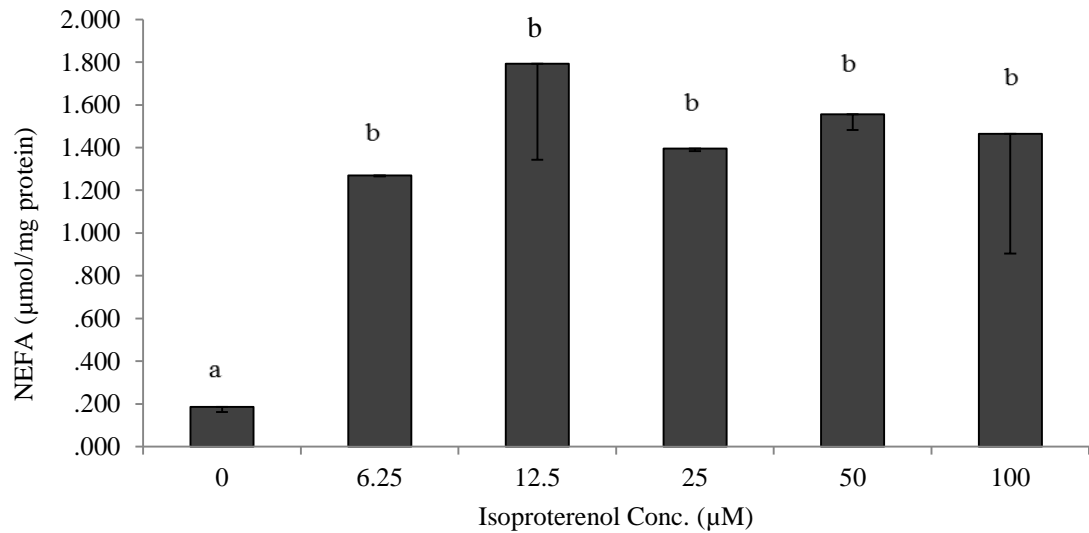


Fig. 3.4 NEFA increased significantly until 6.25  $\mu\text{M}$  isoproterenol, before NEFA concentration plateaued. Mature 3T3-L1 cells were incubated in the presence of increasing doses of isoproterenol for 120 min. Lowercase letters shared in common indicate no significance, data expressed as median and quartiles,  $P \leq 0.05$ .  $n = 3$ .

Glycerol was also measured in the presence of increasing concentrations of isoproterenol after 120 min incubation (see Fig 3.5) and these data exhibit a similar pattern to NEFA. Glycerol concentration did not significantly increase after 12.5  $\mu\text{M}$  isoproterenol, compared with 25  $\mu\text{M}$  ( $P = 0.695$ ); 50  $\mu\text{M}$  ( $P = 0.638$ ) and 100  $\mu\text{M}$  ( $P = 0.437$ ). There was however, a significant increase in glycerol from when exposed to 0  $\mu\text{M}$  to 6.25  $\mu\text{M}$  isoproterenol ( $P = 0.002$ ) and from 6.25  $\mu\text{M}$  to 12.5  $\mu\text{M}$  isoproterenol ( $P = 0.039$ ). There was also a significant increase in concentration of glycerol release after 120 min, between 6.25 and 100  $\mu\text{M}$  isoproterenol ( $P = 0.046$ ). In Fig. 3.6, in comparison with isoproterenol alone, in the presence of 10  $\mu\text{M}$  isoproterenol plus 4 mM acetate, there does not appear to be a change in the concentration of NEFA at any time point over the 240 min incubation.

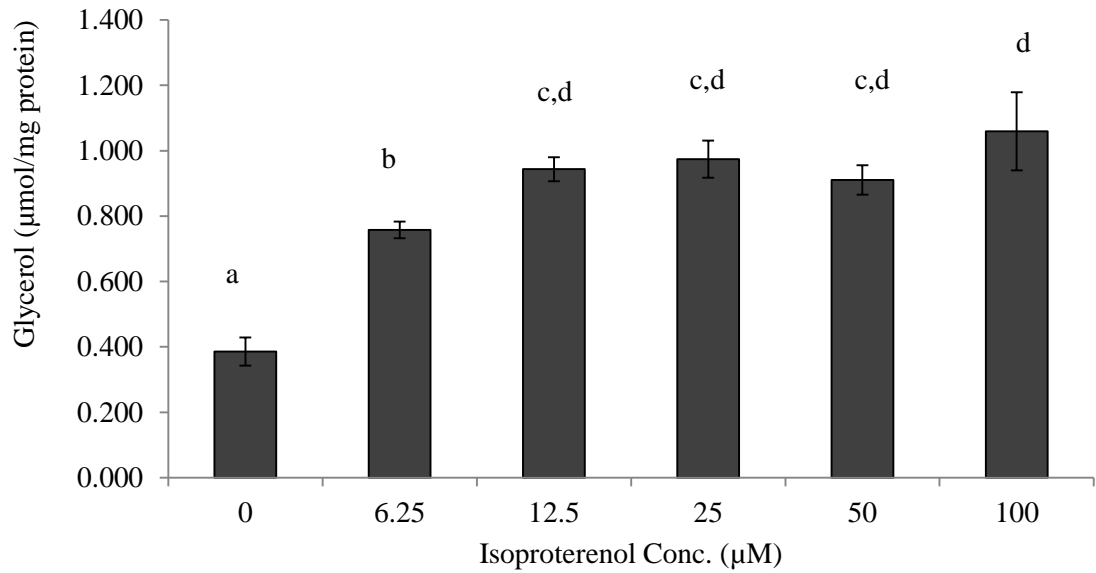


Fig. 3.5 Isoproterenol increased glycerol concentration in a dose dependent manner until ~12.5  $\mu\text{M}$ . Mature 3T3-L1 cells were incubated in the presence of increasing doses of isoproterenol for 120 min. Lowercase letters shared in common indicate no significance  $P \leq 0.05$ .  $n = 3$ .

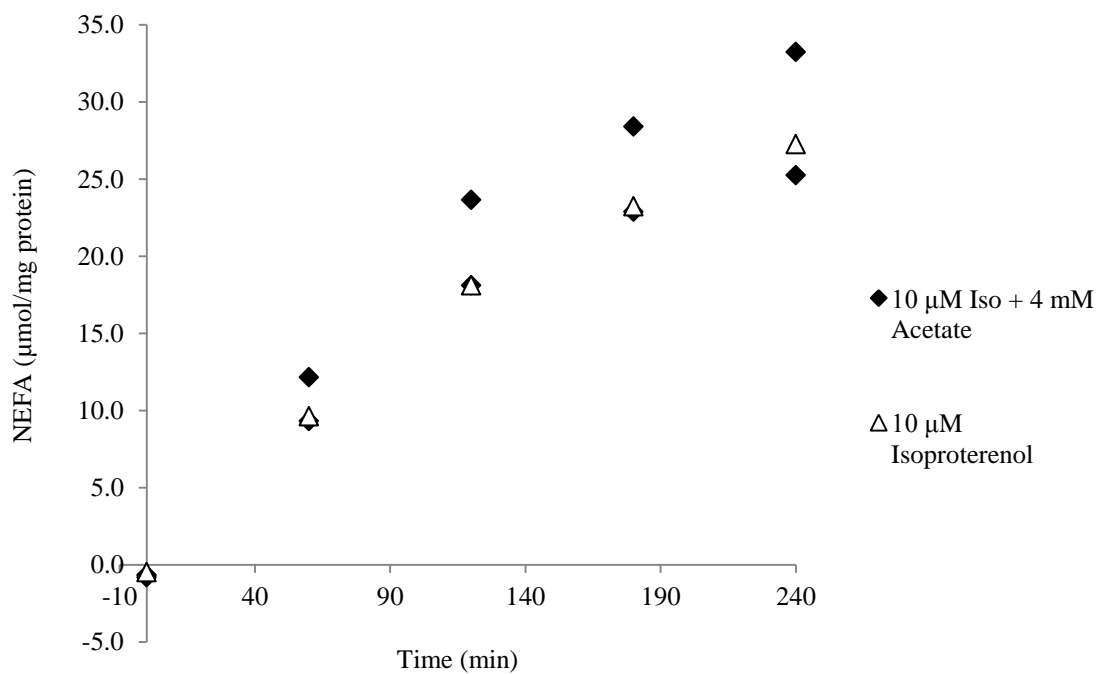


Fig 3.6 3T3-L1 cells stimulated with 10  $\mu\text{M}$  isoproterenol in the presence or absence of 4 mM sodium acetate.  $n = 1-2$ .

### The effect of sodium acetate titration on the change in NEFA and glycerol (3T3-L1 cells)

As shown in Fig. 3.7 at 60 min, sodium acetate did not result in a significant effect on NEFA compared with 5  $\mu$ M isoproterenol alone. However, at 120 min the presence of 4 mM and 6 mM sodium acetate resulted in a significant reduction in NEFA compared with isoproterenol alone ( $P = 0.039$ ;  $P = 0.039$ , respectively). By 180 min, 2, 6 and 8 mM sodium acetate had resulted in a significant reduction in NEFA concentration compared with isoproterenol (2 mM,  $P = 0.05$ ; 6 mM,  $P = 0.05$  and 8 mM,  $P = 0.05$  vs. isoproterenol). The change in glycerol concentration in the media from 3T3-L1 cells in the presence of 2, 4, 6 and 8 mM sodium did not differ significantly from glycerol in the presence of isoproterenol alone at 60, 120 and 180 min (see Fig. 3.8).

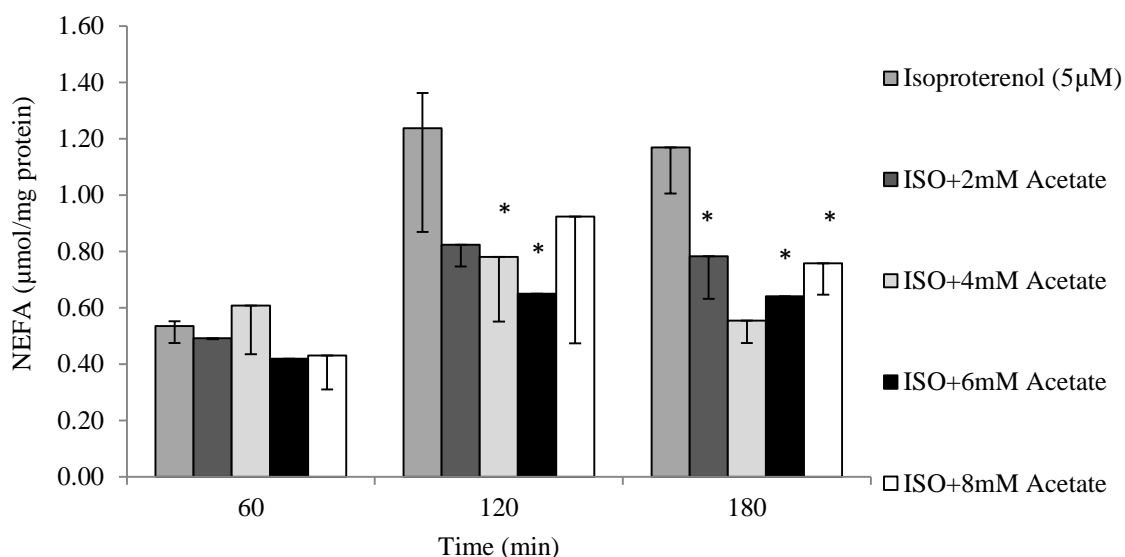


Fig. 3.7 At 120 and 180 min, under stimulated conditions NEFA was reduced in the presence of sodium acetate. Change in concentration was calculated by subtracting 0 min from each subsequent time point. Values are median and quartiles,  $*P < 0.05$  vs. isoproterenol.  $n = 3 - 6$ .

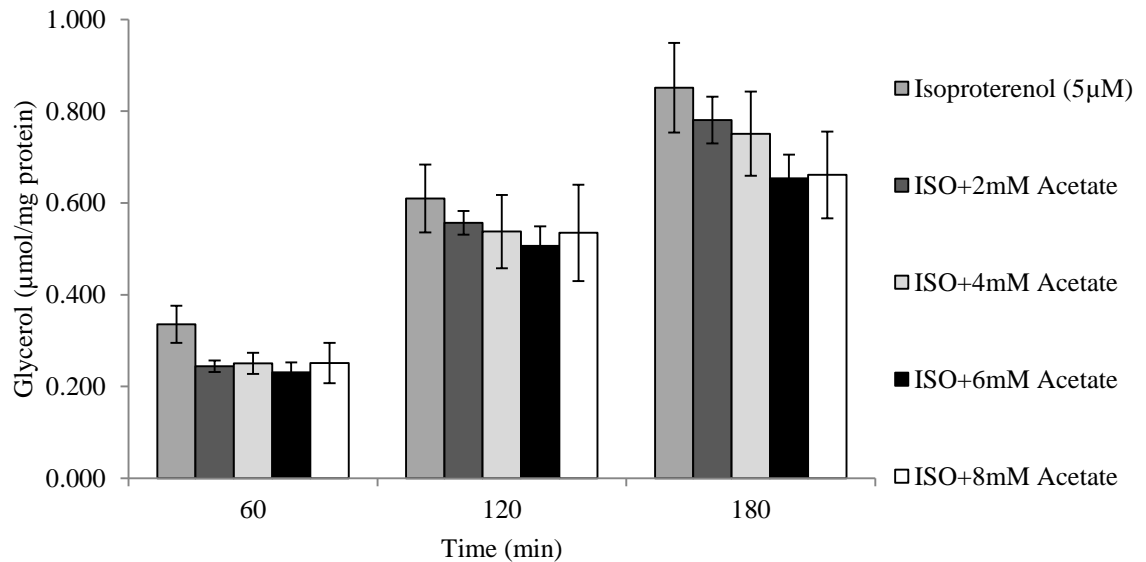


Fig. 3.8 Sodium acetate resulted in a reduction in glycerol in the media from cells incubated under stimulated conditions at 60 min. Change in glycerol was calculated by subtracting 0 min from each subsequent time point. Values are means  $\pm$  SE,  $n = 3 - 6$ .

### The effect of sodium acetate titration on total NEFA and total glycerol in cell media

After calculating the total concentration of NEFA and glycerol during 0 to 180 min sodium acetate treatment inclusive (Trapezoid calculation); 2mM, 4mM, 6mM and 8mM sodium acetate resulted in a significant reduction in NEFA compared with isoproterenol treated cells alone ( $P = 0.016$ ;  $P = 0.019$ ;  $P = 0.003$  and  $P = 0.012$ , respectively), see Fig. 3.9. Due to a lack of significant change in glycerol over time in Fig 3.8, there was also no significant change in total glycerol concentration in the presence of each sodium acetate concentration (see Fig 3.10). Compared with isoproterenol alone, the addition of either 2 mM, 4 mM, 6mM or 8 mM sodium acetate did not significantly reduce total glycerol ( $P = 0.994$ ;  $P = 0.969$ ,  $P = 0.711$  and  $P = 0.688$  vs. isoproterenol, respectively).



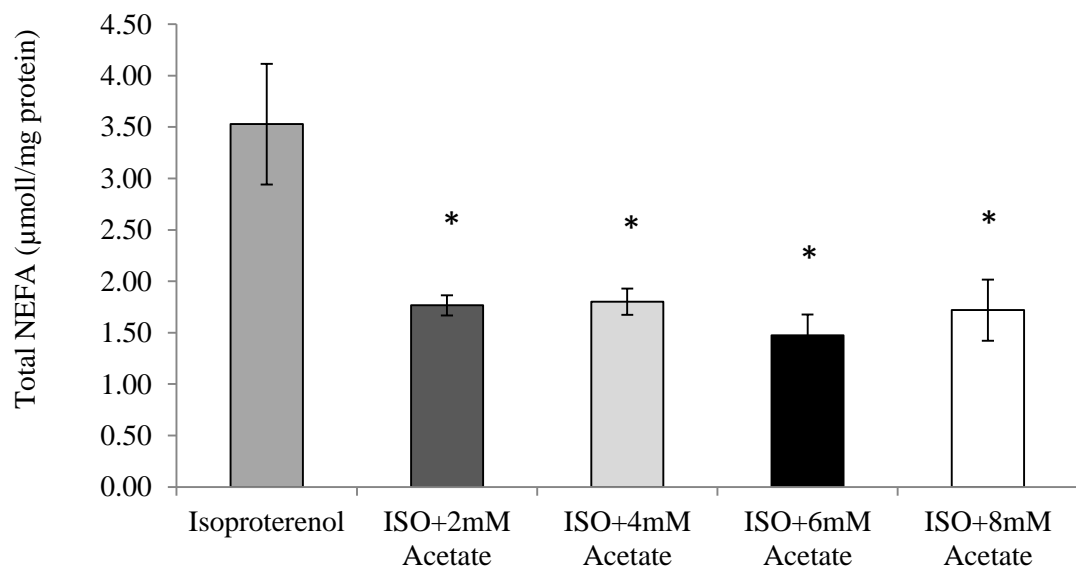


Fig. 3.9 Total NEFA was significantly reduced in the presence of 2, 4, 6, 8 mM sodium acetate compared with isoproterenol alone. Values are means  $\pm$  SE, \* $P$  < 0.05 vs. isoproterenol,  $n$  = 3.

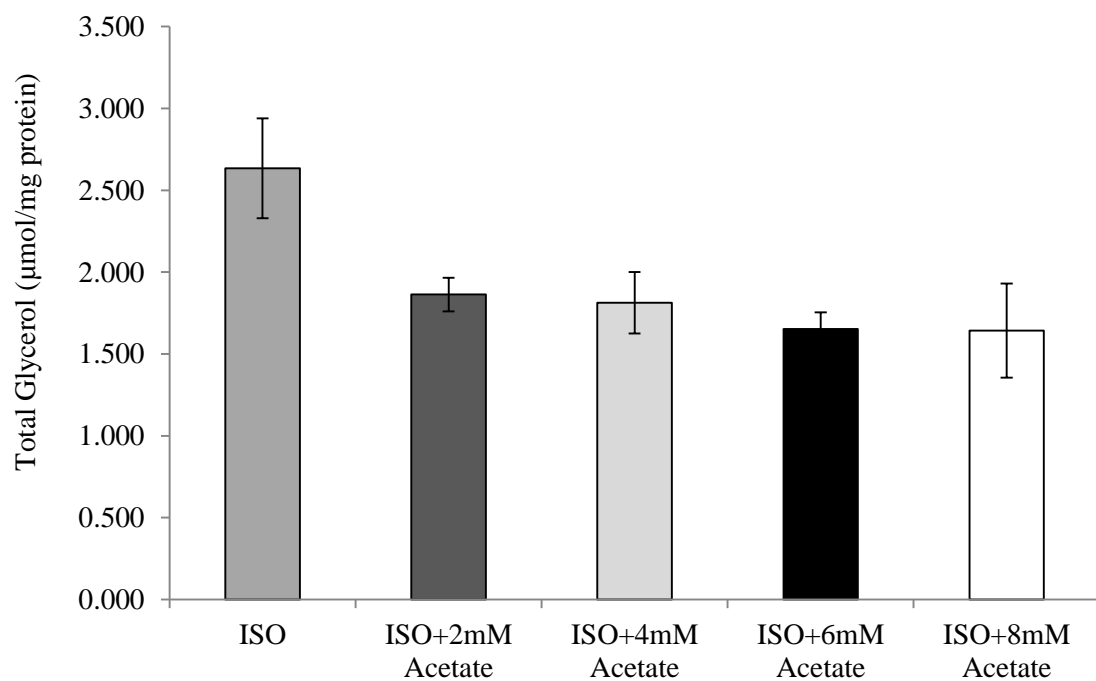


Fig 3.10 The presence of 2, 4, 6 and 8 mM sodium acetate did not reduce total glycerol significantly, compared with isoproterenol alone. Values are means  $\pm$  SE,  $n$  = 3.



## Western blot analysis

### Protein titration

In Fig 3.11, it was apparent that isoproterenol resulted in a substantial increase in phosphorylation of pHSL<sub>(SER563)</sub> as expected, however isoproterenol treatment did not change the level of GAPDH and therefore was unaffected by treatment conditions and could be used as a reliable loading control.

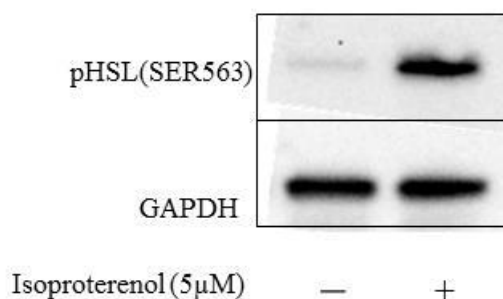


Fig. 3.11 Isoproterenol resulted in an increase in the level of pHSL present in each sample; however isoproterenol did not affect the level of GAPDH.  $n = 1$ .

From Fig 3.12 there appears to be no noticeable difference in pixel signal intensity from GAPDH between 30 and 40  $\mu\text{g}$  protein, but by 50  $\mu\text{g}$  the definition of the image had become less clear. Therefore an acceptable range of protein volume to use in subsequent samples would be 15 - 30  $\mu\text{g}$  protein as the potential for pixel bleaching is minimised at this volume.

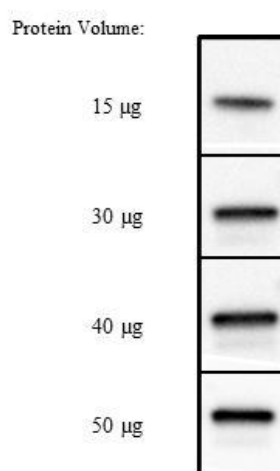


Fig 3.12 Increasing the volume of protein per sample above 40  $\mu\text{g}$  resulted in an increase in the potential for pixel bleaching of GAPDH. Mature 3T3-L1 cells were incubated for 120 min in basal media.  $n = 1$ .

### Temporal resolution of the basal activity of 3T3-L1 cells

Under basal conditions, the highest peak of phosphorylation of HSL occurred at 120 min compared to time 0 as shown in Fig. 3.13. The level of HSL phosphorylation occurring at 150, 180 and 210 appears to have become reduced compared to 0 min, however due to the lack of repeat experiments statistical analysis could not be performed on this data.

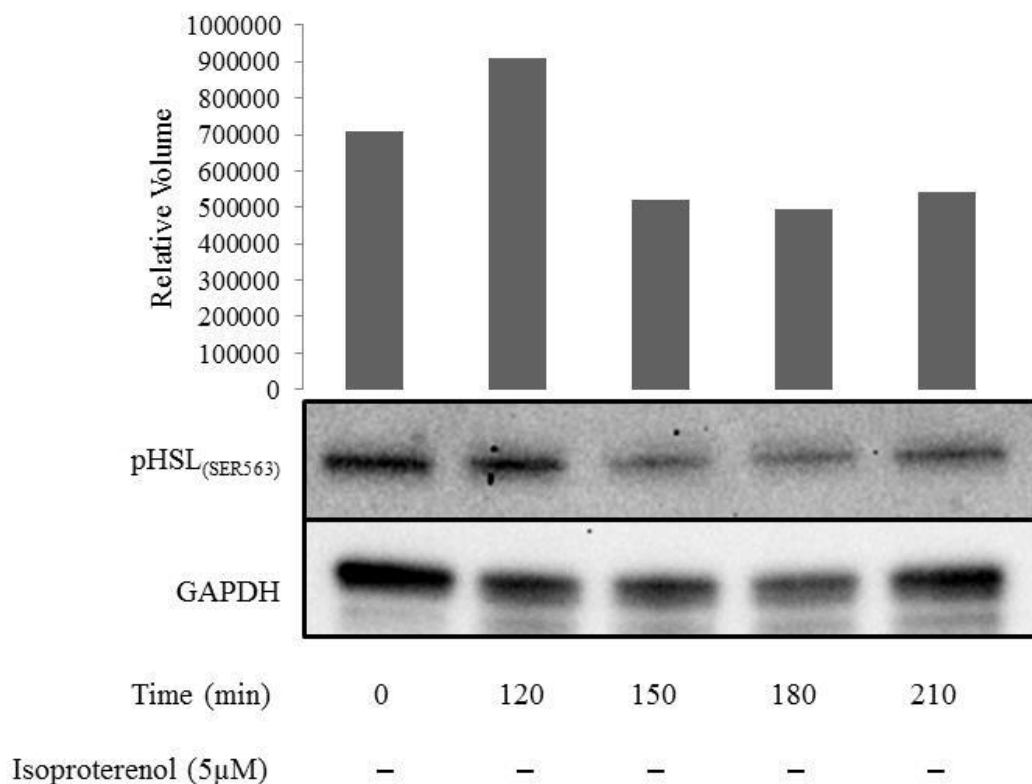


Fig 3.13 Under basal conditions, phosphorylation of HSL occurred at its highest level at 120 min, compared to time 0 min.  $n = 1$ .

### Temporal resolution of the stimulated activity of 3T3-L1 cells

As shown in Fig 3.14, in the presence of isoproterenol (5  $\mu$ M), the relative volume of pixel intensity of pHSL increased  $\sim 8$  fold between 0 min and 60 min incubation. The relative volume of pixel intensity of pHSL decreased  $\sim 25$  % between 60 and 90 min, and by  $\sim 20$  % between 120 and 150 min. After 150 min incubation the level of pHSL is still  $\sim 4.7$  fold higher than that observed at time 0 min. Sample containing 25  $\mu$ g protein was loaded into each well. To ensure that the relative volume of pixel intensity of HSL phosphorylation in each sample was comparable between treatments, all samples were normalised relative to GAPDH,

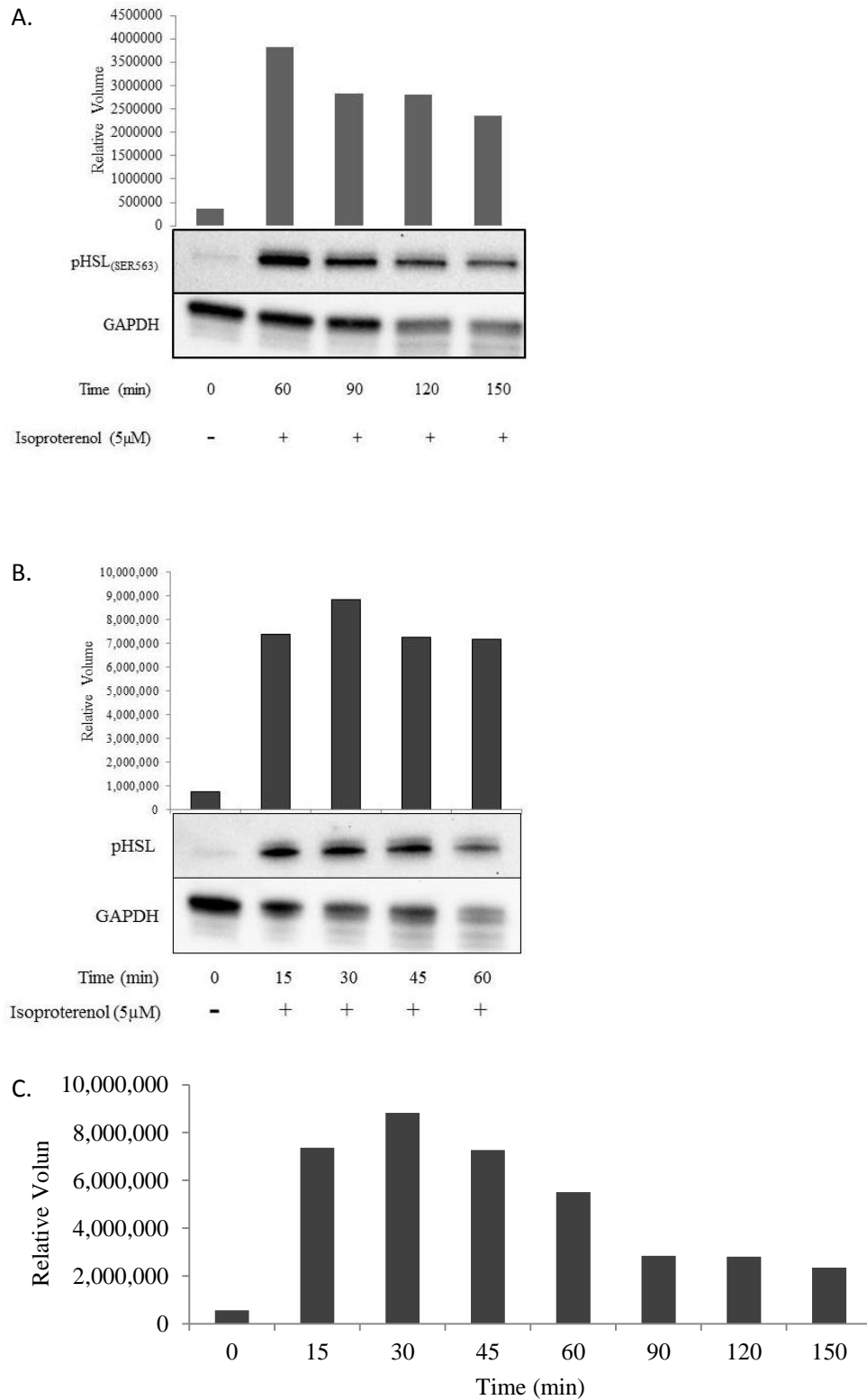


Fig. 3.14 A. Relative volume of pHSL increased by ~90 % in the presence of 5 μM isoproterenol after 60 min incubation. B. After 30 min incubation, relative volume of pHSL increased ~ 91 % in the presence of 5μM isoproterenol. C. Combining data from both A. and B. demonstrates that after 30 min in the presence of isoproterenol (5μM), relative volume of pHSL has increase by ~ 94 %. n = 1 - 2.

### Isoproterenol, acetate and insulin interference with NEFA assay (in the absence of 3T3-L1 cells)

In Fig. 3.15, the effects of basal media spiked with either acetate, insulin or NaAc+Ins., did not result in a significant difference in NEFA concentration compared with basal media alone ( $P = 0.244$ ,  $P = 0.182$  and  $P = 0.609$  respectively). As shown in Fig. 3.16, Isoproterenol, Iso+Acetate, Iso+Insulin and Iso+NaAc.+Ins. also did not cause a significant change in NEFA concentration compared with basal media alone ( $P = 0.311$ ,  $P = 0.082$ ,  $P = 0.219$  and  $P = 0.185$  vs. basal respectively).

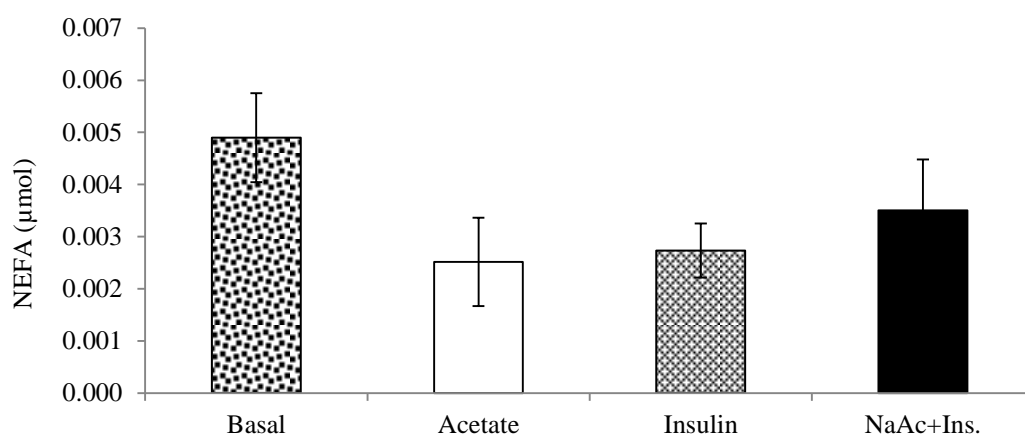


Fig. 3.15 In basal medium, the presence of acetate and NaAc+Ins., did not result in a significant change in the background absorbance of the NEFA assay. Data are Mean  $\pm$  SE, \* $P < 0.05$ ,  $n = 4 - 7$ .

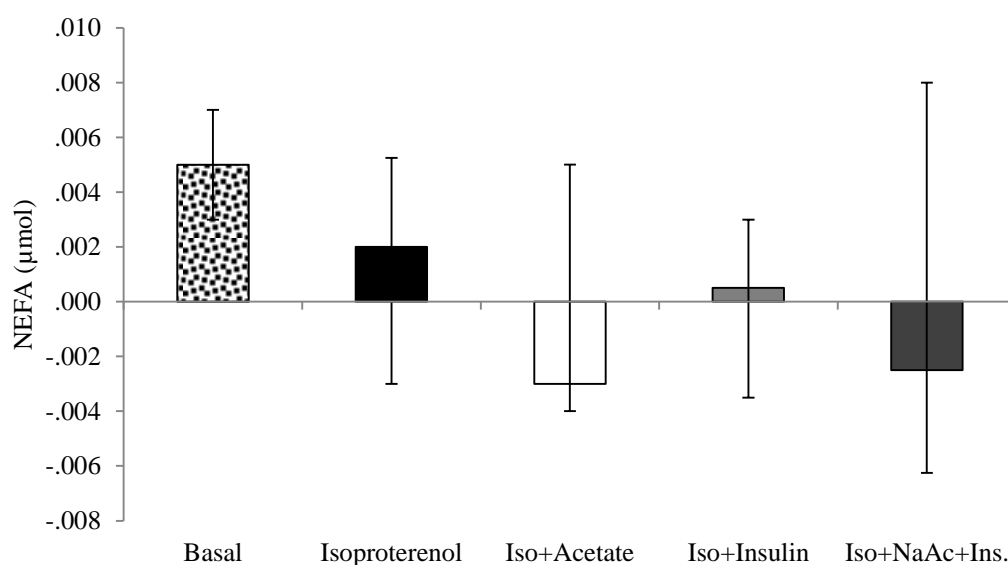


Fig. 3.16 In basal medium, the presence of isoproterenol, acetate, insulin and NaAc+Ins. did not result in a significant change in background absorbance of the NEFA assay. Data are Median and quartiles,  $n = 4 - 8$ .

### NEFA assay interference over 120 minutes

It would appear that the treatment compounds, acetate and insulin, do not interfere with the baseline NEFA concentration found in media collected from isoproterenol stimulated cell alone, after a 120 min incubation (See Fig. 3.17). Samples were spiked with acetate and insulin before measurement of NEFA concentration.

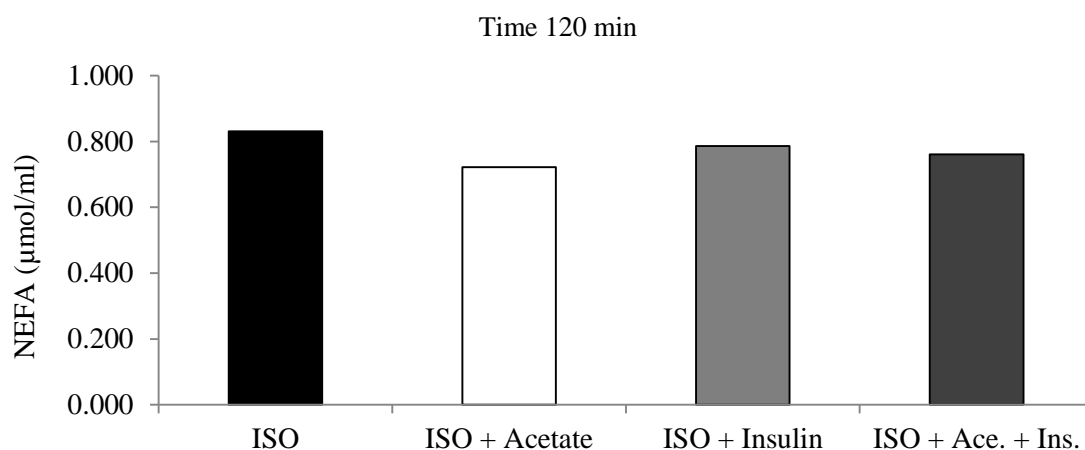


Fig. 3.17 Treatment compounds did not interfere with NEFA assay after 120 min incubation. Stimulated cell media was spiked with or without sodium acetate (4mM) and with or without insulin (1.34 μM). Due to a lack of repeat experiments, statistical analysis could not be performed. n = 1.

### The effect of lysis buffer interference on total protein calculation (in the absence of 3T3-L1 cells)

From the results in Fig. 3.18, it is apparent that in its concentrated form, the 10x stock solution of lysis buffer resulted in a ~ 4.6 fold increase in the absorbance reading for the total protein reagent (Coomasie Blue) compared to sterile water at absorbance 595nm. The concentration recommended by the manufacturer was a 1x solution; however, this concentration also resulted in a ~3.5 fold background reading of absorbance compared to water. In order for the cell suspension and pellet samples collected in subsequent studies to be analysed within the linear element of the BSA standard curve, all samples were diluted a further 10 fold in sterile water. To replicate these conditions a 10 fold dilution of the 1x lysis buffer was analysed and was found to have a background absorbance similar to that found in sterile water (see Fig. 3.19.).

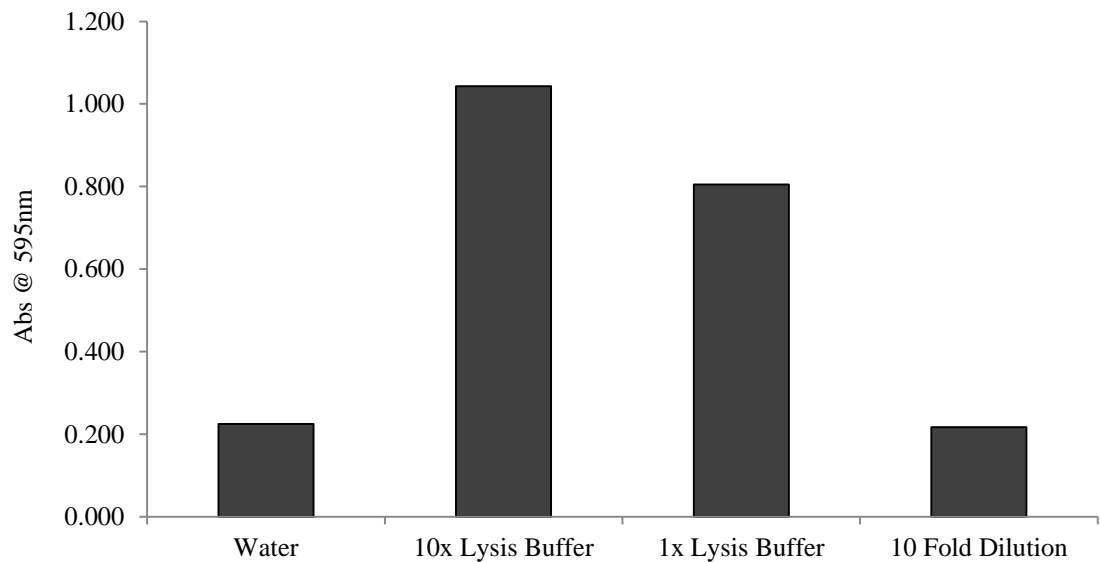


Fig. 3.18 Cell lysis buffer at the concentration used during total protein analysis did not interfere with the absorbance reading at 595 nm. n = 1.

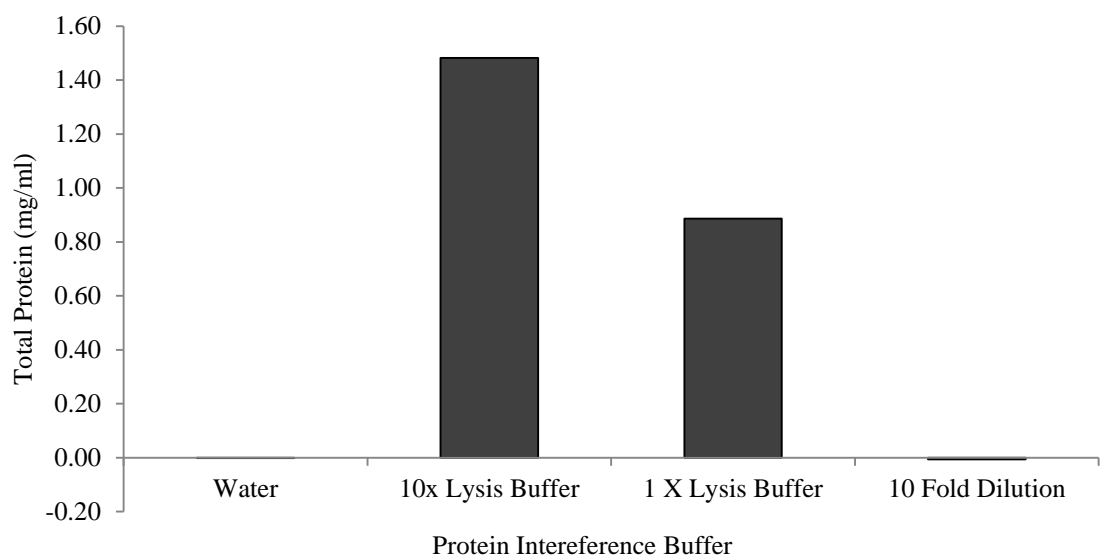


Fig. 3.19 Cell lysis buffer did not interfere with total protein calculation when diluted 10 fold in sterile water. n = 1.

### Primary cell isolation – Cytotoxicity

As shown in Fig. 3.20A, after 4 hrs incubating in three different combinations of digestion buffer (DB1, DB2 and DB3); cells isolated with both collagenase + liberase (DB3) were most viable, illustrated by a change in the cell media from blue to pink illustrating a change in resazurin (blue) to resorufin (pink) (O'Brien et al., 2000). In Fig. 3.20 B, after a 6 hr incubation, both DB3 wells 1 and 2 had changed from blue to pink.

In the presence of DB1, collagenase alone, the cells were able to metabolise resazurin to resorufin after 20 hrs post-isolation (Fig. 3.20 C). However, cells isolated in DB2 did not show any sign of metabolism, as cell media containing resazurin remained blue at all three time points (Fig 3.20 A,B,C). As expected cells incubated in Triton-X100 for 1 hr did not metabolise resazurin to resorufin and therefore remained blue. Also, after 20 hrs incubation, in Fig. 3.20C, DB3 well 1 had become colourless as resorufin was finally metabolised to hydroresorufin..

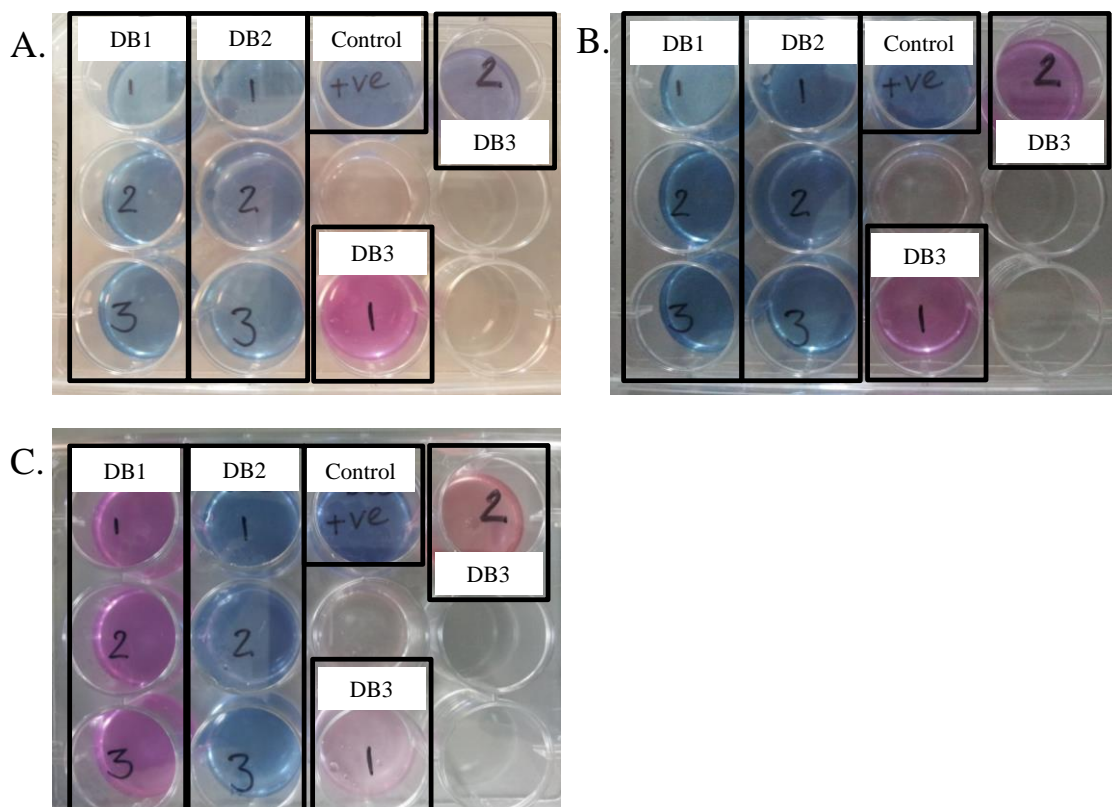


Fig. 3.20 Measure of cell cytotoxicity after dissection and isolation of mature primary adipocytes. A. 4 hr post isolation. B. 6 hr post isolation. C. 20 hr post isolation. Cells were isolated in 3 various digestion buffers; DB1 Collagenase (1mg/ml); DB2 Liberase (0.15 Wünsch units); DB3 Collagenase (1 mg/ml) + Liberase (0.15 Wünsch units). n = 2 - 3.

## Discussion

There were many reasons for conducting these studies, and one of the first was to understand the physiology of the 3T3-L1 cell line, including the maturation process of lipid droplet formation. The second was to ascertain whether the proposed treatment conditions would result in interference with the metabolic assays. And finally, studies using techniques such as western blotting were conducted to map any changes in protein expression over time and to confirm the protein GAPDH as a loading control.

From the literature, it was identified that 3T3-L1 cells could be utilised for experiments at different stages of cell maturity, evidenced by the use of mature cells on different days post-differentiation (Ge et al., 2008, Cao et al., 2007). It is understood that this particular cell line can be used between days 7 and 14 post-differentiation (Zen-Bio, 2012); and it is clear in Fig. 3.1, that the level of TAG incorporated into lipid droplets increases greatly before this time, Fig. 3.1 A-D. Due to the increase in lipid accumulation within lipid droplets observed in Fig 3.1 A-D, the number of days post-differentiation cells are experimented on, may impact on the results obtained. Therefore, all subsequent studies involving the use of 3T3-L1 cells were conducted on the same day (9 days post-differentiation). Also, as shown in Fig. 3.5 and Fig. 3.6, the concentration required to stimulate lipolysis by 100 % was established for isoproterenol in both the glycerol and NEFA assays. It was apparent from the data that ~10  $\mu$ M isoproterenol was required to stimulate lipolysis by 100 %, a concentration commonly used by others (Rodríguez et al., 2011, Lasa et al., 2012). From the results it was apparent that there was not a linear relationship between the dose required to stimulate NEFA and glycerol release. Therefore the EC<sub>50</sub> (concentration required to stimulate a reaction by 50 %) could not be calculated. From data in Fig. 3.6 in the presence of 10  $\mu$ M isoproterenol, 4 mM acetate was unable to reduce NEFA release, therefore, for subsequent experiments it was suggested that a submaximal concentration of isoproterenol be used. For this reason a concentration of 5  $\mu$ M would be used.. It was also identified that the supernatant fraction of the cell lysate contained soluble protein as measured by the Bradford reagent technique. All subsequent tests were conducted with the inclusion of both pellet and supernatant fractions when normalising by-products of lipolysis (NEFA, glycerol, TAG) to total protein. From the literature, studies have also reported data collected after the administration of a range of concentrations of acetate (Ferchaud-Roucher et al., 2005, Nuutinen et al., 1985, Nilsson and Belfrage, 1978), therefore as sodium acetate was the treatment of interest, a dose



response relationship in the presence of 5  $\mu$ M isoproterenol was also completed. In the presence of 2, 4, 6 and 8 mM sodium acetate, NEFA was measured every 60 min for 3 hrs. From the results shown in Fig. 3.9, after 180 min incubation, total NEFA was significantly reduced by all concentrations of sodium acetate (2, 4, 6 and 8mM); however, as shown in Fig 3.7, at 120 min, only 4 mM and 6 mM sodium acetate reduced NEFA significantly. For this reason 4 mM sodium acetate was used in all subsequent experiments. It should also be noted that this concentration of acetate would be regarded as supra-physiological, and would not occur naturally in humans. Therefore, all subsequent data should be analysed on the understanding that the effects observed would only occur in circumstances where acetate had been artificially increased, for investigation into therapeutic targets.

Many factors influence the reliability of the western blot technique e.g. gel running and transfer times, blocking time and specificity of antibodies. Firstly, as seen in Fig. 3.11, GAPDH was chosen as a loading control as it was unaffected by the treatment of cells with isoproterenol. Secondly, it was important to identify the most appropriate concentration of protein to use, as seen in Fig. 3.12. Finally, as the metabolic studies were conducted over a 180 min incubation, an appropriate time point to stop treatment incubation for the purpose of western blot analysis was chosen. From Fig. 3.13, under basal conditions, pHSL phosphorylation was highest at 120 min; however there was no data for earlier time points. The data in Fig. 3.14 C, under stimulated conditions, however, showed that pHSL peaked at 30 min and decreased until 90 min where phosphorylation appeared to plateau. Metabolic sampling at 0, 60, 120 and 180 min had concluded prior to the start of western blot analysis. Therefore, to allow for comparison at the same time point between metabolic samples and protein expression of pHSL<sub>(SER563)</sub>, western blot analysis would also be conducted on samples that had been incubated under basal and stimulated conditions for 120 min.

As the parameters for cell treatment were now identified it was important to check that the compounds of interest would not interfere with the metabolic assay used to identify changes in adipocyte lipolysis. Data in Fig. 3.15 and 3.16 illustrate that treatment with isoproterenol, acetate and insulin either alone or in combination would not interfere with the NEFA assay, regarding changes in background absorbance. Similarly, samples collected after 120 min (Fig. 3.17), from cells incubated in the presence of isoproterenol, which were subsequently spiked with treatments including acetate and insulin, did not interfere with NEFA assay. There is also literature on the potential

interference of buffers on total protein analysis (Mohan, 1997). Compounds used within some lysis buffers e.g. Tris Buffers, have been identified as having the potential to interfere with the sensitivity of assays including the Bradford assay for total protein calculation. This was also true for the 3T3-L1 lysis buffer as shown in Fig. 3.18 and Fig. 3.19. However, it was identified that a 10-fold dilution of the recommended 1 x solution did not interfere with total protein calculation and so all subsequent samples containing the 1 x lysis buffer were diluted 10 fold before protein analysis.

The effects of isolation of primary adipocytes on cell viability were also considered. It was important to get a high yield of viable cells, but also ensure the least number of animals were used to obtain this. There are a number of published protocols for the isolation of mature murine adipocytes (Rodbell, 1964, Hausman et al., 2008) however, the exact details associated with digestion buffers is limited. As seen in Fig. 3.20, it was the DB containing both collagenase and liberase (DB3) that isolated the most viable cell population.

Finally, consideration should also be given to the validity of other experiments which will not be covered in this thesis, but could also be of relevance. Changes in NEFA and glycerol release are a reliable way of determining the effect of SCFA acetate availability on adipocyte lipolysis, but also experiments focusing on the activation of SCFA receptor GPR43 could also complement these data. The utilisation of known inhibitors of Gi linked receptors, such as pertussis toxin (Ge et al., 2008), would elucidate the involvement of GPR43 activation in the presence of supra-physiological concentrations of acetate. Similarly, while pHSL<sub>(SER563)</sub> will be analysed as a marker for intracellular changes to lipolysis, consideration of the analysis of total HSL and changes to pHSL<sub>(565)</sub> would also support the data acquired in this thesis, regarding a potential mechanism of action.

Overall, from the analysis of initial experimental methods and from the hypotheses made, it can be concluded that all treatment compounds either individually or in combination do not interfere with the NEFA assay to any significant extent. A concentration of 4 mM sodium acetate would be used in subsequent studies, as opposed to the hypothesised concentration of 2 mM, even though this would be considered supra-physiological. This was due to the findings that 4 mM acetate was the lowest concentration tested to consistently inhibit NEFA concentration in the media over time, and therefore should be considered as a potential therapeutic target depending on the

results of the following studies. As hypothesised,  $\sim 10\ \mu\text{M}$  of the  $\beta$ -AR agonist isoproterenol stimulated the concentration of NEFA and glycerol in the media by 100 %, however, as the relationship of the dose response was not linear, an exact  $\text{EC}_{50}$  could not be calculated. Also, from other data, it was clear that  $10\ \mu\text{M}$  isoproterenol masked the effects of  $4\ \text{mM}$  acetate and so a sub-maximal concentration of isoproterenol,  $5\ \mu\text{M}$ , would be used in all subsequent experiments. For western blot analysis, GAPDH was unaffected by treatment of isoproterenol as hypothesised and pHSL concentration increased in stimulated cells compared to the basal state. Finally, all subsequent primary adipocyte isolation will be performed using DB3, as this combination produced cells with least cytotoxicity, and therefore most viable.

## **Chapter 4**

### **The effect of sodium acetate on white adipose cell basal lipolysis**

## Introduction

As mentioned previously in Chapter 1, adipocyte lipolysis is the pathway responsible for the breakdown of intracellular TAG into glycerol and NEFA. Regulation of this pathway involves the integration of many extracellular and intracellular signalling cascades, including SNS and insulin sensing to the cell surface, followed by lipolysis and lipogenesis within the cell itself. To maintain homeostasis within the mature adipocyte, many substrates and hormones e.g. insulin, norepinephrine and cAMP, interact in a reciprocal way to either stimulate or inhibit lipolysis depending on the energy status of the cell. These interactions occur simultaneously to ensure tight regulation of TAG availability.

In the case of lipolysis, fatty acids hydrolysed from the glycerol backbone of TAG not only exit the cell into the surrounding plasma for use in other cells as an energy supply, but many become re-esterified back into TAG (Jensen et al., 2001). However, under conditions including the post-prandial state, there is a sufficient supply of glucose to the cells and therefore the need for free-fatty acid production is reduced. To ensure there is a reduction in NEFA release and increased uptake of available glucose, insulin acts via IR within the cell surface of adipocytes. Insulin not only regulates uptake of glucose, initiating translocation of GLUT4 transporters from intracellular stores to the plasma membrane, but also inhibits lipolysis through the phosphatidylinositol 3' kinase (PI3-Kinase) dependent pathway and subsequent activation of PDE3B (Chaves et al., 2011, Cheatham et al., 1994). Endogenous regulators such as insulin and norepinephrine are not the only influence on lipolysis, as there is evidence to suggest nutritional substrates also affect the metabolic function of lipolysis, including SCFA (Metz et al., 1974, Al-Lahham et al., 2010, Ge et al., 2008, Taggart et al., 2005). The three predominant SCFA include acetate, butyrate and propionate, which are found in the circulation after fermentation of fibrous foods in the gut by microflora, or metabolism of alcohol in the liver, and therefore effects on stimulated AT lipolysis have been noted previously (Nilsson and Belfrage, 1978, Bernalier-Donadille, 2010, Siler et al., 1999, Ferchaud-Roucher et al., 2005). However, there is a reduced volume of literature on the effects of SCFA availability on basal lipolysis.

Many metabolic studies conducted on the mature adipocyte cell line 3T3-L1 utilise endogenous or artificial stimulation of lipolysis e.g. epinephrine or isoproterenol (Daval et al., 2005, Hong et al., 2005, Mulder et al., 2005), however, it is understood that basal

lipolysis occurs in the absence of external stimulation. A study by Nilsson and Belfrage (1978), demonstrated that in the basal state, glycerol release from isolated rat adipocytes was not significantly reduced in the presence of 10 mM acetate. Furthermore, Morimoto et al. (1998) demonstrated a lack of insulin induced reduction in free fatty acid release from adipocytes isolated from non-fasted 8 and 10 week old rats. In contrast, a study by Miyoshi et al. (2008) identified that stimulation of ATGL was important in basal lipolysis, as measured by a decrease in glycerol release in an ATGL knockdown models. As the exact mechanism of action of acetate on adipocyte lipolysis is still unknown, it was hypothesised that in the presence of supra-physiological concentration of sodium acetate (4 mM), basal lipolysis would be not be decreased, in the form of NEFA and glycerol, and that there would be no additive effect in the presence of both insulin and sodium acetate.

## Methods

Briefly, on the day of sample collection, day 9 post differentiation, murine 3T3-L1 cells were washed in 37°C 1 x PBS before being placed in treatment media (DMEM plus 2 % bovine serum albumin – fatty acid free (BSA-FFA)). Treatment conditions were prepared using 4 mM sodium acetate and 1.34  $\mu$ M insulin, in the following combinations 1. Basal 2. Acetate 3. Insulin and 4. NaAc+Ins.. For sodium acetate treatments, cells were pre-incubated in the presence of 4 mM acetate for 30 minutes prior to PBS cell wash step. Samples were collected every 60 minutes over a maximum period of 180 minute. After the last sample was collected, cells were lysed and sonicated before being centrifuged at 10,000 x g for 10 minutes at 4°C. Media samples were stored at -20°C and supernatants were stored at -80°C. NEFA, glycerol, TAG and total protein samples were analysed as described in Chapter 2. Total NEFA and total glycerol ( $\mu$ mol/mg protein) were calculated using the area under the curve for each replicate from 0 to 180 min, before calculating the mean and standard error or median and quartiles for each treatment condition.

## Statistical analysis

All statistical analysis was performed using IBM SPSS Statistics 20 software. All data sets were checked for normality using the Shapiro-Wilk test as there were < 50 samples per data set, and a boxplot was used to check for outliers. Data found to be normally distributed were analysed using a 1-way ANOVA followed by post-hoc Tukey test. Paired non-parametric data were analysed using a Friedmans test followed by pair-wise Wilcoxin. For un-paired data that were also non-parametric, data were analysed using Kruskal-Wallis followed by pair-wise Mann Whitney test. A *P* value of  $\leq 0.05$  was considered statistically significant.

## Results

### The effect of 4 mM sodium acetate and insulin on basal NEFA in the media from 3T3-L1 cultured mature adipocytes

Under basal treatment conditions NEFA increased from 0 to 180 min, with 0.005  $\mu\text{mol}/\text{mg}$  protein at 0 min, 0.089  $\mu\text{mol}/\text{mg}$  protein at 60 min, 0.200  $\mu\text{mol}/\text{mg}$  protein at 120 min and 0.289  $\mu\text{mol}/\text{mg}$  protein by 180 min. In the presence of acetate, insulin and NaAc+Ins. there was no statistical change in NEFA compared with basal accumulation at time 0, 60 and 120 min. However, at 180 min, in the presence of acetate, NEFA concentration in the media was significantly reduced compared with NEFA in the presence of basal treatment alone ( $P = 0.004$ ).

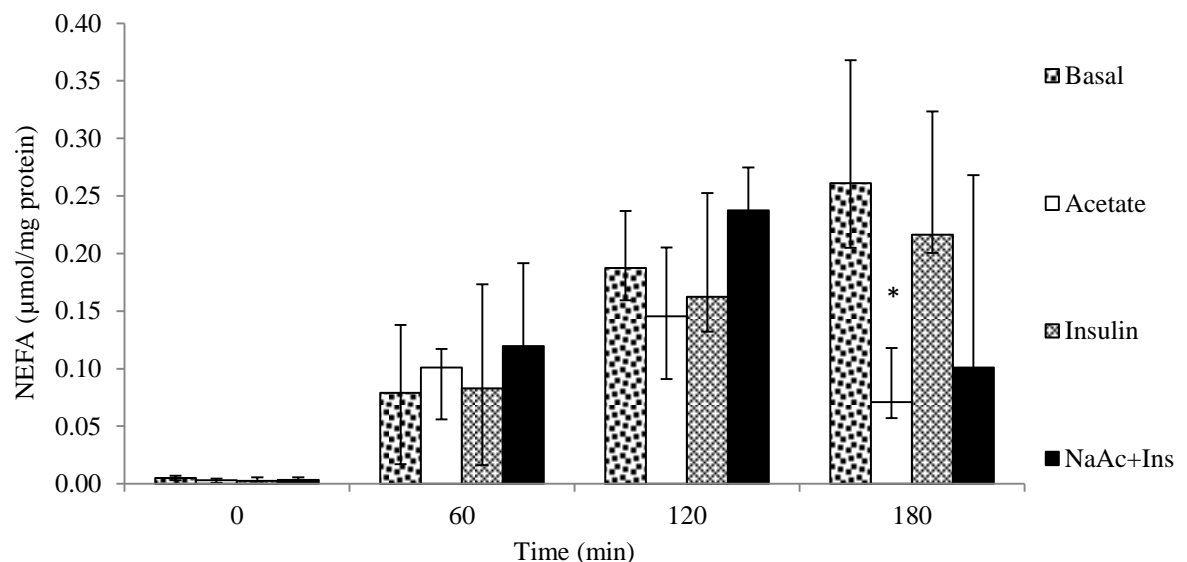


Fig. 4.1 4 mM sodium acetate reduced the concentration of NEFA in the media from 3T3-L1 cells after 180 min incubation. Values are Median and Quartiles,  $*P \leq 0.05$ .  $n = 4 - 7$ .

### Total basal NEFA concentration over 180 minutes

The total concentration of NEFA in the media in the presence of acetate alone (0.30  $\mu\text{mol}/\text{mg}$  protein) was not significantly reduced compared with basal treatment alone (0.43  $\mu\text{mol}/\text{mg}$  protein), as shown in Fig. 4.2 ( $P = 0.462$ ), as calculated by area under the curve. In the presence of insulin and NaAc+Ins. treatments there was also no significant change in total NEFA compared with basal concentration alone ( $P = 0.530$  and  $P = 0.986$ , respectively).



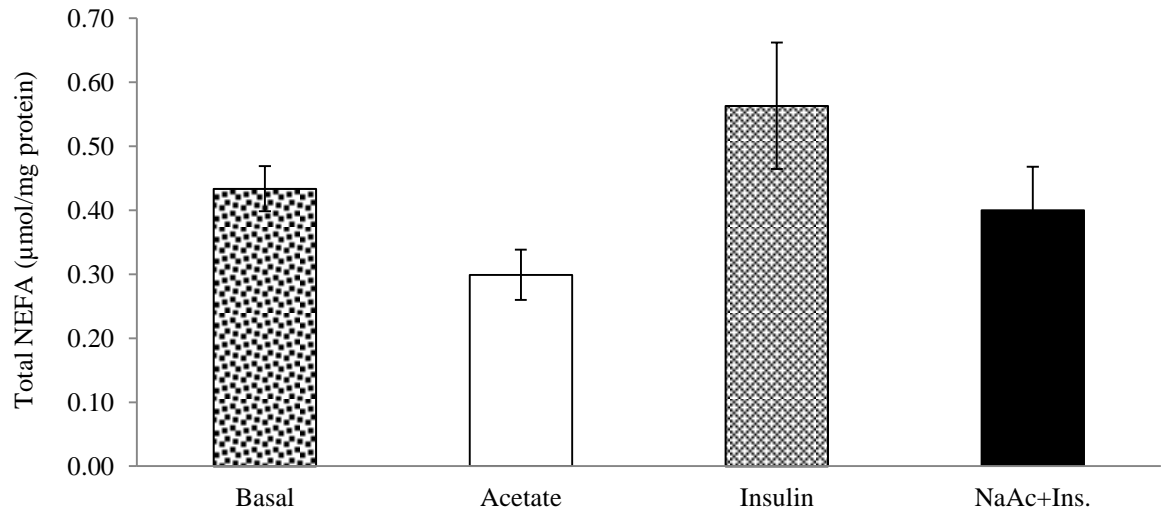


Fig. 4.2 4 mM sodium acetate does not result in a significant decrease in total NEFA over 180 min compared with total basal NEFA in the media. The total concentration of NEFA is expressed as mean  $\pm$  SE.  $n = 3 - 5$ .

#### **The effect of 4 mM sodium acetate and insulin on basal glycerol in the media from 3T3-L1 cultured mature adipocytes**

There was no significant change in glycerol in the media when comparing basal treatment with acetate, insulin and NaAc+Ins at time 0 min ( $P = 0.387$ ,  $P = 0.990$  and  $P = 0.985$ ) (Fig. 4.3). The background concentration of glycerol at time 0 min in basal, acetate, insulin and NaAc+Ins. media was found to be, 0.43  $\mu\text{mol/mg protein}$ , 0.55  $\mu\text{mol/mg protein}$ , 0.42  $\mu\text{mol/mg protein}$  and 0.46  $\mu\text{mol/mg protein}$ , respectively. The concentration of glycerol in the media at 180 min had increased significantly compared with glycerol concentration at 0 min in basal ( $P = 0.000$ ) and insulin ( $P = 0.002$ ) treated cells, however, when comparing 0 min to 180 min glycerol concentration in the presence of acetate and NaAc+Ins., there was no significant change ( $P = 0.349$  and  $P = 0.340$ , respectively). Within each time point, 0, 60, 120 and 180 min, there was also no significant difference in glycerol concentration when comparing basal treatment with acetate, insulin and NaAc+Ins (0 min  $P = 0.317$ ; 60 min  $P = 0.279$ ; 120 min  $P = 0.946$  and 180 min  $P = 0.210$ ), see Fig. 4.3.

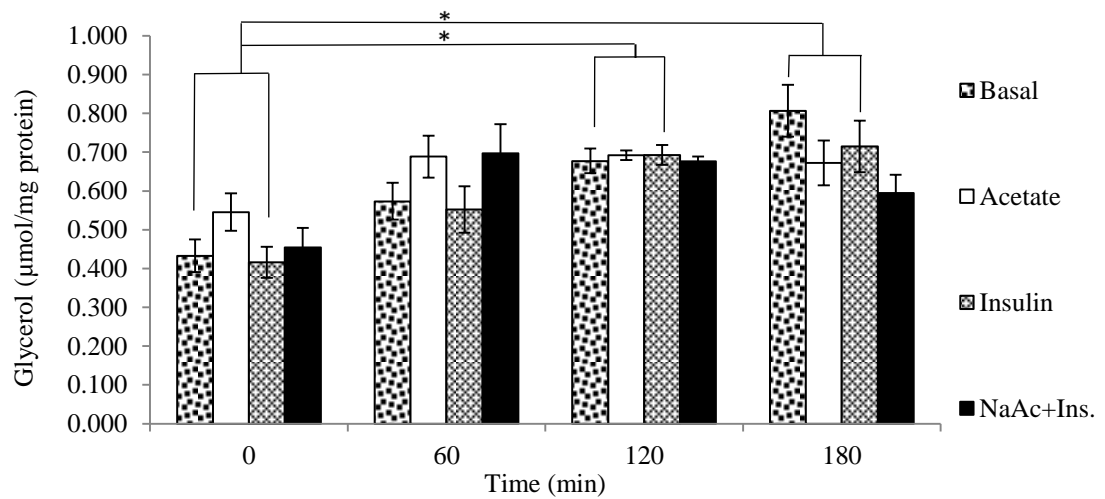


Fig. 4.3 4 mM sodium acetate did not alter glycerol in the media compared with basal treatment in 3T3-L1 cells during 180 min incubation. Values are mean  $\pm$  SE,  $*P \leq 0.05$ .  $n = 2 - 6$ .

#### Total basal glycerol concentration over 180 minutes

As shown in Fig. 4.4, there was no change in the total concentration of glycerol in any treatment condition compared with total basal glycerol alone (acetate  $P = 0.961$ ; insulin  $P = 1.000$  and NaAc+Ins.  $P = 0.999$  vs. basal).

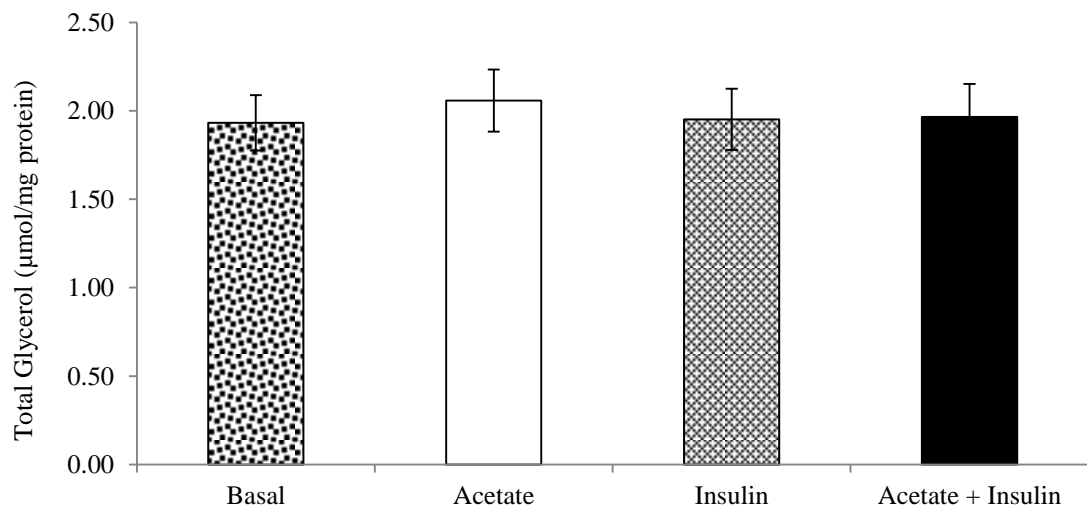


Fig. 4.4 4 mM sodium acetate did results in a significant decrease in the total concentration of glycerol in the media over 180 min compared with total basal glycerol. The total concentration of glycerol is expressed as mean  $\pm$  SE.  $n = 3 - 5$ .

### Adjusted media glycerol concentration under basal lipolysis conditions in 3T3-L1 cells

Due to the high background level of glycerol present in the treatment media (Fig. 4.3), it was important to subtract this from each subsequent time point. This allowed for identification of the true level of glycerol present in the media in each treatment condition over time. As shown previously (Fig 4.3), there were no statistical differences in glycerol accumulation between acetate, insulin, NaAc+Ins and basal treatments at 0 min. Therefore any differences in glycerol at subsequent time points (60, 120 and 180) could not be attributed to compound interference with the assay. Furthermore, after subtraction of time 0 min glycerol, as shown in Fig. 4.5, adjusted media glycerol concentration after 60 min incubation had increased significantly in the presence of NaAc+Ins. compared with basal treatment ( $P = 0.039$ ). This significant change had been abolished by 120 min, as neither acetate, insulin nor NaAc+Ins. treatment resulted in a significant change in glycerol at this time point (acetate  $P = 0.064$ , insulin  $P = 1.000$  and NaAc+Ins.  $P = 1.000$ ). However, by 180 min, acetate has significantly reduced glycerol ( $0.127 \mu\text{mol/mg protein}$ ) compared with basal treatment ( $0.374 \mu\text{mol/mg protein}$ ) ( $P = 0.020$ ). Also, NaAc+Ins. had significantly reduced glycerol in the media by 180 min ( $0.140 \mu\text{mol/mg protein}$ ), compared with basal treatment alone ( $P = 0.020$ ).

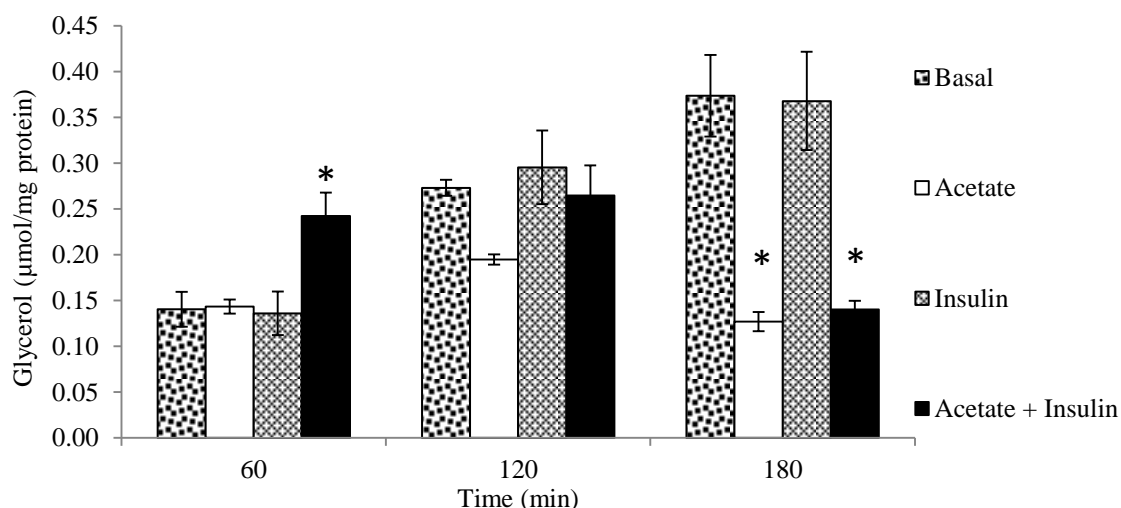


Fig. 4.5 In the presence of sodium acetate, adjusted glycerol concentration was reduced after 180 min incubation. Concentration of glycerol at 0 min was subtracted from each subsequent time point to calculate change in glycerol. Values are means  $\pm$  SE, \* $P < 0.05$  vs. basal.  $n = 2 - 6$ .

### The effect of 4 mM sodium acetate and insulin on intracellular TAG in 3T3-L1 cultured mature adipocytes under conditions of basal lipolysis

There were no significant differences in intracellular TAG after 180 min incubation with each treatment ( $P = 0.719$ ), as seen in Fig. 4.6. Compared with basal treatment alone, incubation with acetate, insulin and NaAc+Ins. for 180 min, did not result in a significant reduction in intracellular TAG ( $P = 1.000$ ,  $P = 0.745$  and  $P = 0.999$ , respectively).

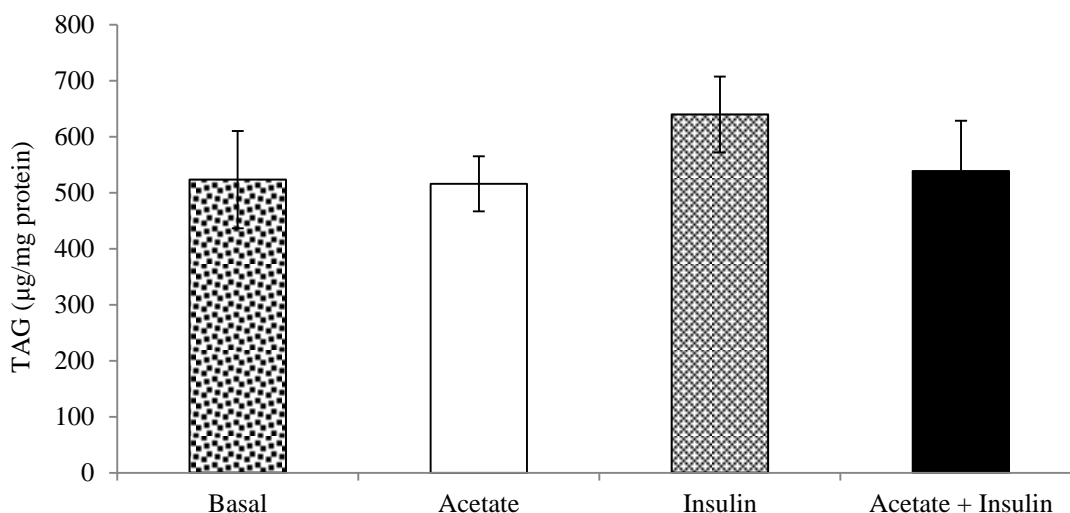


Fig. 4.6 4 mM Sodium acetate did not result in a significant decrease in intracellular TAG after 180 min incubation compared with intracellular TAG in basal treatment, mean  $\pm$  SE.  $n = 3 - 6$ .

## Discussion

From the results of this study, it is possible to conclude that a supra-physiological concentration of sodium acetate does have a small inhibitory effect on the metabolic function of adipocytes, contrary to our original hypothesis. This was evident through a reduction in both NEFA and glycerol media concentration after a 180 min incubation. However, total NEFA and total glycerol remained unchanged compared with control, in the presence of 4 mM acetate and 1.34  $\mu$ M insulin. Intracellular TAG levels also remained statistically unchanged in basal (un-stimulated) treatment conditions. In agreement with the hypothesis, it would appear that at a concentration of 1.34  $\mu$ M insulin was unable to reduce lipolysis, and therefore, there was no evidence for an additive effect in the presence of both sodium acetate and insulin at these concentrations.

All treatment conditions were compared to basal concentrations of NEFA at 60 min intervals over the 180 min incubation. In the presence of 4 mM acetate, the accumulation of NEFA in the media was significantly decreased after 180 min compared with basal treatment conditions. . However, as shown in Fig. 4.1, the only time point where the presence of acetate resulted in a significant reduction in NEFA media accumulation was in the final 60 min incubation. Furthermore, at a concentration of 1.34  $\mu$ M, insulin did not reduce basal NEFA or glycerol at any time point over the 180 min incubation. Similar to these data, a study by Morimoto et al. (1998) demonstrated no change in FFA release in the presence of  $10^{-6}$  M insulin. Furthermore, it should also be noted that the concentration of insulin used in this study would be described as physiologically high, as post-prandial concentrations of insulin reported by Poretsky (2010), only reach concentrations of around 350 pM. There appeared to be a high background of glycerol present in the 0 min samples. This may have been due to the composition of the treatment media, as although it was possible to control the level of background NEFA with BSA-FFA, we were unable to do the same for glycerol. Over 180 minutes there was a steady increase in the concentration of glycerol into the surrounding media from treated cells, however, total glycerol (Fig. 4.4) did not appear to vary significantly between treatments. However acetate, insulin and NaAc+Ins. treatments did not significantly change total glycerol compared with basal conditions. Similarly, data reported in the study by Nilsson and Belfrage (1978) demonstrated minimal or no reduction in glycerol release in the presence of 10 mM acetate in the basal state. Consideration should be given to the hypothesis that glycerol accumulation

in the media did not significantly alter over time due a lack of concentration gradient. With such a high background concentration of glycerol and supra-physiological concentrations of insulin in the media, it could be possible that hydrolysed glycerol after lipolysis could not exit the cell via aquaglyceroporins. (Carbrey et al., 2003) noted that AQP9, a common hepatic glycerol channel, expression was down-regulated in the presence of insulin compared with control. However, subtraction of the background concentration of glycerol from subsequent time point data in this thesis demonstrated significant changes in glycerol in the presence of each treatment. In the presence of acetate, change in glycerol concentration decreased significantly at 180 min, however acetate had no effect at 60 and 120 min. The change in glycerol concentration in the presence of insulin showed there was no significant reduction at either 60, 120 or 180 min. NaAc+Ins. treatment was most unusual as results showed a significant increase in glycerol concentration at 60 min; however a significant reduction by 180 min compared to basal treatment. As the data from total NEFA and glycerol were not significantly different in the presence of all treatment conditions compared with the basal state, levels of intracellular TAG were also likely to remain unchanged. At 180 minutes the data collected for intracellular TAG were as expected. The small concentration of NEFA and glycerol d from the cells may account for a lack of significant change in total intracellular TAG. However, it was noted during the protocol that the method for TAG isolation may introduce a certain level of human error. This is due to the solidification of TAG during the centrifugation process occurring at 4°C, and subsequent clumping of TAG on microtube walls. Other methods of analysing TAG would be more relevant to this study, including organic extraction, or radiolabelling TAG for analysis before and after treatment. Also, one limitation identified from these data, is a lack of re-uptake data for both NEFA and glycerol, therefore it is only possible to draw conclusions on the accumulation of NEFA and glycerol in the media and not on actual release. This can be confirmed from the data, as it would appear in Fig. 4.1 that NEFA accumulation in the media went down between 120 and 180 min. This could be accounted for by a re-uptake of NEFA into the cell for use either in mitochondria or as a substrate for lipogenesis. .

Overall, it would appear that complementary to previously reported data, insulin does not significantly reduce free fatty acid accumulation in the media in the basal state, even at a supra-physiological concentration. A concentration of 4 mM acetate was found to reduce glycerol and NEFA concentration after a 180 min incubation to a significant

degree. However, when considering total NEFA, total glycerol and intracellular TAG, acetate was found to have no effect compared with basal treatment alone.

## **Chapter 5**

### **The effect of sodium acetate on white adipose cell stimulated lipolysis**



## Introduction

Over the last 10 years there has been increased interest in the effect of nutritional substrates, such as SCFA, on the metabolic pathway of lipolysis in white adipose tissue (Ge et al., 2008, Hong et al., 2005). As part of this, surface receptor activation and the lipolytic pathway, including glycerol and NEFA accumulation in the cell media have been reviewed in great detail (Lafontan and Langin, 2009, Duncan et al., 2007).

Activation of the  $\beta$ -AR is an integral part in regulation of lipolysis in the presence of catecholamines and other peptide hormones i.e. norepinephrine, insulin and glucagon etc. (Mulder et al., 2005, Nakamura, 2006, Collins et al., 2004). The  $\beta$ -ARs are located in the cell membrane of mature adipocytes, and the receptor found in highest abundance in adipose tissue is  $\beta$ 3 (Collins and Surwit, 2001). There can be either a stimulatory or inhibitory GPCR linked to the surface  $\beta$ -AR receptor (Rasmussen et al., 2011). The binding of ligands to the surface receptor, and the coupling of a stimulatory G protein, initiates activation of adenylyl cyclase, increased availability of cAMP, subsequent PKA phosphorylation of HSL and perilipin and finally hydrolysis of stored TAG into diacylglycerol, monoacylglycerol, before releasing NEFA and glycerol (Ahmadian et al., 2009). Under condition of inhibitory G protein coupling,  $G_{\alpha i}$ , the initial adenylyl cyclase activation is blunted and therefore reduces the amount of available cAMP to promote TAG hydrolysis.

There have been many  $\beta$ -ARs found within the plasma membrane of mature adipocytes, including the small human carboxylic acid receptor HM47A, prostaglandin receptors EP3 and PGE2 and the inhibitory G protein-coupled receptor GPR43, which has been identified as a receptor for SCFA (Ge et al., 2008, Le Poul et al., 2003). Reasons for the presence of a specific receptor for SCFA within white adipose tissue have not been fully identified, but it does suggest that SCFA may play a part in regulation of adipocyte lipolysis. A previous *in vivo* human study by Smith et al. (2007), observed that plasma concentrations of NEFA and glycerol did not rise after the consumption of sodium acetate. The study aimed to identify the effects of acetate metabolism on fat and carbohydrate utilisation. The authors administered either sodium acetate or sodium bicarbonate, both of which induced a metabolic alkalosis, where it was observed that both NEFA and glycerol concentration increased in the first hour after sodium bicarbonate ingestion but that sodium acetate ingestion did not result in a change in NEFA and glycerol, and there was a concomitant decrease in fat oxidation. The authors

concluded that acetate did suppress lipolysis during a metabolic alkalosis, i.e. conditions that stimulated lipolysis (Smith et al., 2007). As the changes observed in the study by Smith et al. (2007), were conducted *in vivo*, utilisation of the 3T3-L1 cell line would give more insight into the mechanism(s) of action of sodium acetate on stimulated adipocytes *in vitro*.

Therefore, due to our understanding of GPR43 activation by SCFA, it has been hypothesised that incubation with 4 mM acetate will reduce  $\beta$ -AR stimulated phosphorylation of HSL at the serine 563 residue compared to control. Furthermore, as it is widely documented in the literature that activation of the insulin receptor results in cAMP reduction to 5'AMP by PDE3B, we hypothesize that treatment of 3T3-L1 adipocytes with 1.34  $\mu$ M insulin in the stimulated state will also result in a decrease in pHSL<sub>(SER563)</sub>. As both inhibitory GPCR and insulin receptor activation have been found to reduce overall lipolysis, it is also hypothesised that NEFA and glycerol, and intracellular TAG will all be reduced compared with  $\beta$ -AR stimulation alone.

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## Methods

Briefly, on the day of sample collection, day 9 post differentiation, murine 3T3-L1 cells were washed in 37°C 1 x PBS before being placed in treatment media. Treatment conditions were prepared using 5  $\mu$ M isoproterenol, 4 mM sodium acetate and 1.34  $\mu$ M insulin, in the following combinations 1. Isoproterenol 2. Iso+Acetate 3. Iso+Insulin and 4. Iso+NaAc+Ins.. For sodium acetate treatments, cells were pre-incubated in the presence of 4 mM acetate for 30 minutes prior to PBS cell wash step. Samples were collected every 60 minutes over a maximum period of 180 minute. After the last sample was collected, cells were lysed and sonicated before being centrifuged at 10,000 x g for 10 minutes at 4°C. Media samples were stored at -20°C and supernatants were stored at -80°C. NEFA, glycerol, TAG and total protein samples were analysed as described in Chapter 2.

## Statistical analysis

All statistical analysis was performed using IBM SPSS Statistics 20 software. All data sets were checked for normality using the Shapiro-Wilk test for data sets with < 50 samples and with Kolmogorov-Smirnov test for data sets with > 50 samples, and boxplots were used to check for outliers. Data found to be normally distributed were analysed using a 1-way ANOVA followed by post-hoc Tukey test. Paired non-parametric data were analysed using a Friedmans test followed by pair-wise Wilcoxin. For un-paired data that were also non-parametric, data were analysed using Kruskal-Wallis followed by pair-wise Mann Whitney test. A *P* value of  $\leq 0.05$  was considered statistically significant.

## Results

### The effect of 4 mM sodium acetate on the phosphorylation of hormone sensitive lipase<sub>(SER563)</sub> in 3T3-L1 adipocytes

In order to identify if sodium acetate and insulin were acting within the lipolytic cascade, changes in the phosphorylation of HSL<sub>(SER563)</sub> were analysed after a 120 min incubation. In the presence of 5  $\mu$ M isoproterenol, the relative volume of pHSL<sub>(SER563)</sub> increased ~2.5 fold compared with the basal state of pHSL<sub>(SER563)</sub>, i.e. without stimulation (Fig. 5.1). Incubating the cells with the addition of Iso+Acetate resulted in the relative volume of pHSL<sub>(SER563)</sub> being reduced by 15 % compared with isoproterenol stimulation alone. pHSL was also reduced in the presence of Iso+Insulin by 21 % and Iso+NaAc.+Ins. by 24 % compared with isoproterenol stimulation.

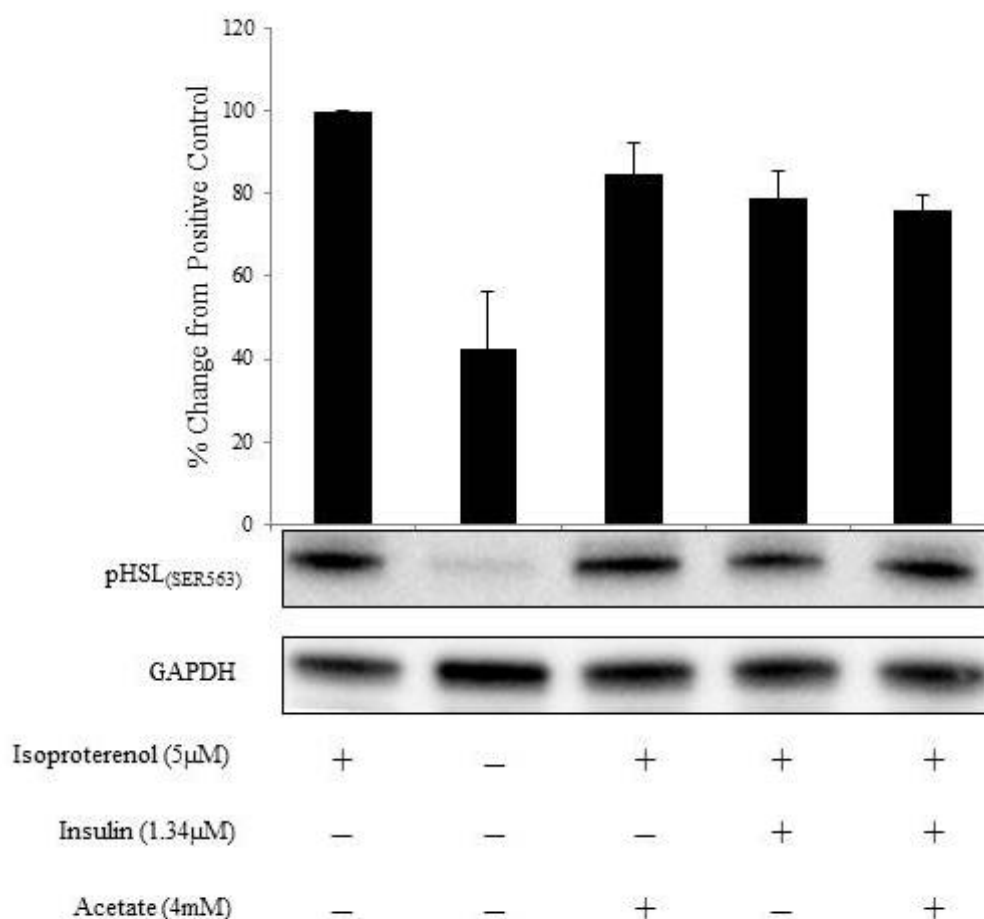


Fig. 5.1 Sodium acetate and insulin resulted in a reduction in the phosphorylation of HSL<sub>(SER563)</sub> in the presence of isoproterenol. Cell lysates were analysed by Western Blot and relative volume was calculated using ImageLab 3.0 normalised to GAPDH. n = 4.

### The effect of 4 mM sodium acetate and insulin on the concentration of NEFA in the media from stimulated 3T3-L1 cultured mature adipocytes

As shown in Fig. 5.2, in the presence of Iso+Acetate, the concentration of NEFA was significantly reduced at both 60 min and 180 min ( $P = 0.004$  and  $P = 0.02$ , respectively) compared with 5  $\mu$ M isoproterenol treatment alone. At 120 min however, Iso+acetate treatment did not reduce NEFA compared with isoproterenol treatment alone ( $P = 0.078$ ).

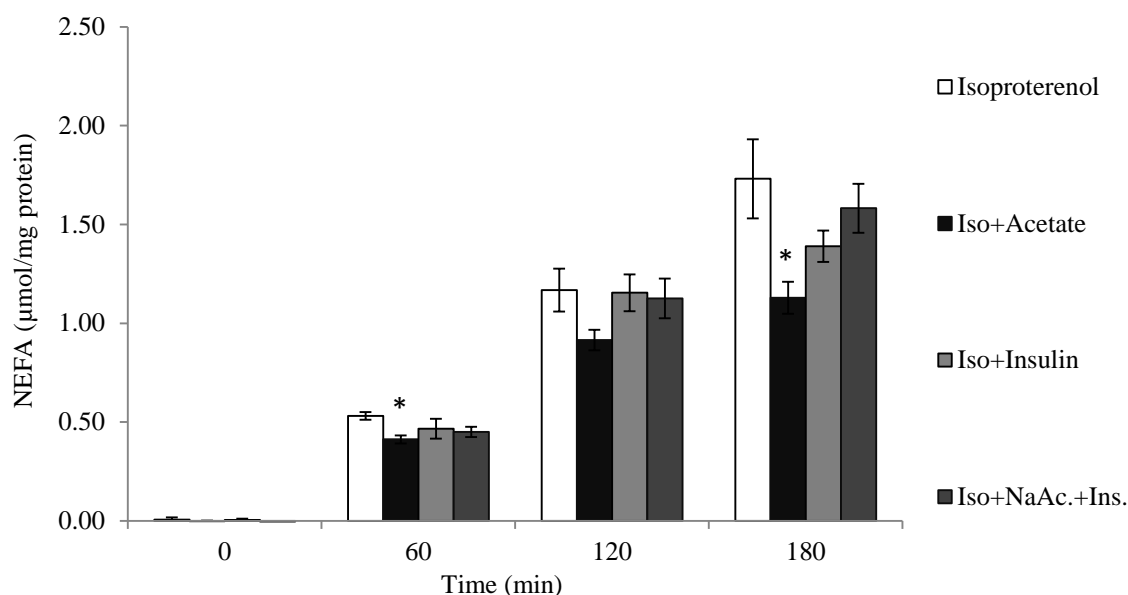


Fig. 5.2 Sodium acetate resulted in a reduction in NEFA in the presence of isoproterenol at 60 and 180 min. Values are means  $\pm$  SE, \* $P < 0.05$  vs. isoproterenol.  $n = 3 - 8$ .

### Total stimulated NEFA concentration over 180 minutes

Total NEFA concentration ( $\mu$ mol/mg protein) was calculated as the AUC of NEFA over 180 minutes. As expected, total NEFA concentration (See Fig. 5.3) followed the same pattern as seen in Fig. 5.2. In the presence of Iso+Acetate there was a 42 % reduction in the accumulation of total NEFA in the media compared with 5  $\mu$ M isoproterenol treatment alone ( $P = 0.025$ ). However, Iso+Insulin and Iso+NaAc+Ins. did not reduce total NEFA significantly ( $P = 0.142$  and  $P = 0.101$ , respectively).

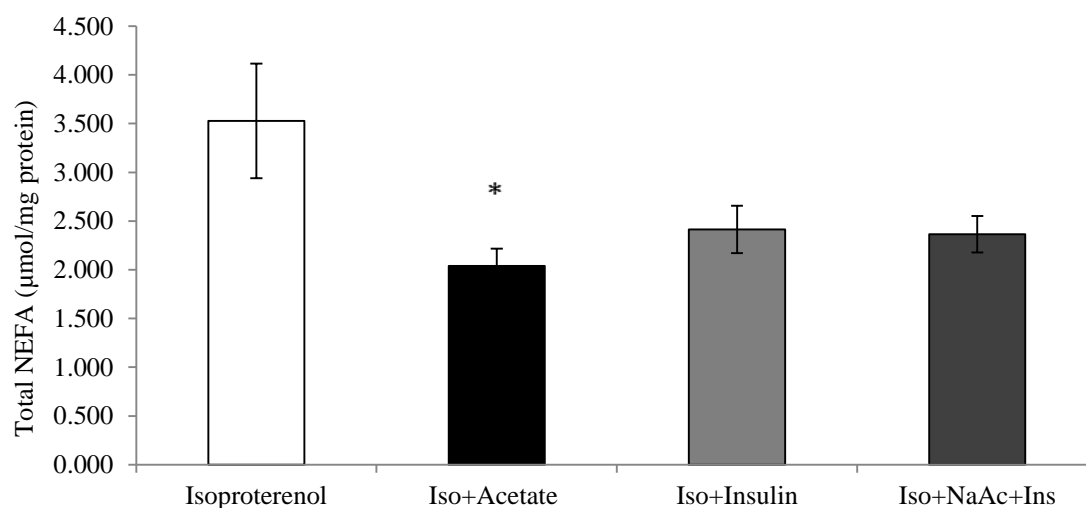


Fig. 5.3 Total stimulated NEFA concentration calculated over 180 minutes under each treatment condition. Total NEFA was obtained by calculating the area under the curve for each replicate treatment condition. Values are mean  $\pm$  SEM, \* $P = 0.053$ ,  $n = 3-5$ .

#### **The effect of 4 mM sodium acetate and insulin on the concentration of glycerol in the media from stimulated 3T3-L1 cultured mature adipocytes**

As shown in Fig. 5.4, in the presence of isoproterenol and Iso+Acetate treatments there was a steady and significant increase of glycerol in the media from 0 - 60 min ( $P = 0.028$  and  $P = 0.028$ , respectively), 60 - 120 min ( $P = 0.027$  and  $P = 0.028$ , respectively) and 120 - 180 min ( $P = 0.028$  and  $P = 0.028$ , respectively). However, Iso+Insulin and Iso+NaAc+Ins. treatments did not result in a significant increase in glycerol at each time point over the 180 min incubation. Unlike the NEFA results, there was no reduction in glycerol concentration in the presence of Iso+Acetate at any time point compared with 5  $\mu$ M isoproterenol alone (0 min,  $P = 0.631$ ; 60 min,  $P = 0.423$ ; 120 min,  $P = 0.749$  and 180 min,  $P = 0.529$ ). This was also the case for Iso+Insulin and Iso+NaAc+Ins. treatments compared with isoproterenol alone, at time points 0 min ( $P = 0.808$ ); 60 min ( $P = 0.172$ ); 120 min ( $P = 0.345$ ) and 180 min ( $P = 0.139$ ). Due to the composition of the treatment media, it was difficult to eliminate all traces of glycerol at time 0 min, accounting for the high background level of glycerol at this time point.

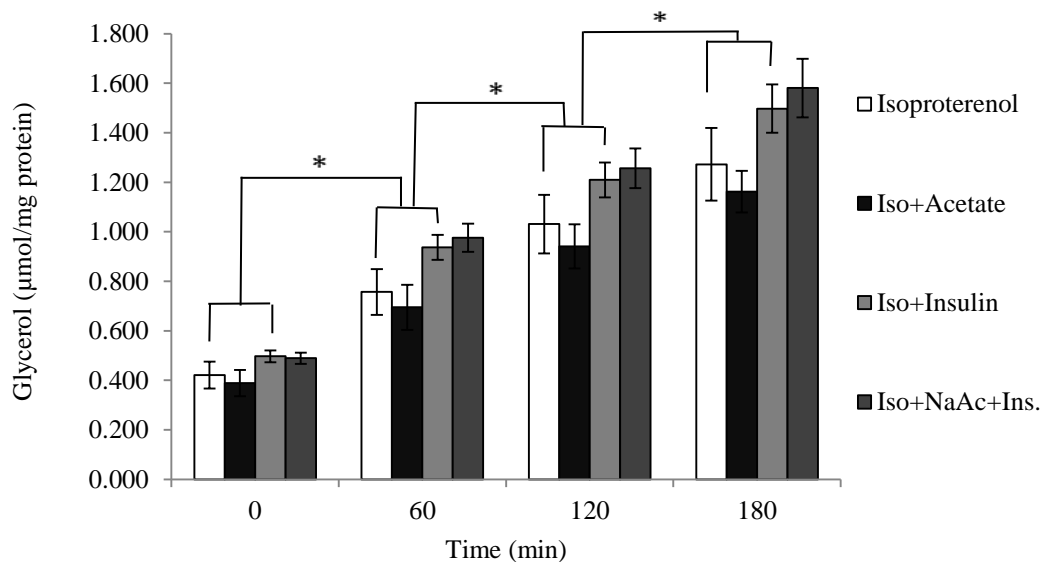


Fig. 5.4 Sodium acetate does not results in a reduction in glycerol in the media in the presence of isoproterenol. Values are means  $\pm$  SE, \* $P < 0.05$  vs. isoproterenol. n = 3 - 6.

### Total stimulated glycerol concentration over 180 minutes

Total glycerol was calculated using the area under the curve in the same way as total NEFA. In the treatment conditions that contained insulin, both Iso+insulin and Iso+NaAc+Ins. did not significantly change the total glycerol concentration compared with 5  $\mu$ M isoproterenol alone (Fig 5.5), ( $P = 0.302$  and  $P = 0.439$ , respectively). Furthermore, treatment with Iso+Acetate did not result in a significant reduction in the total glycerol concentration compared with isoproterenol treatment alone ( $P = 0.262$ ).

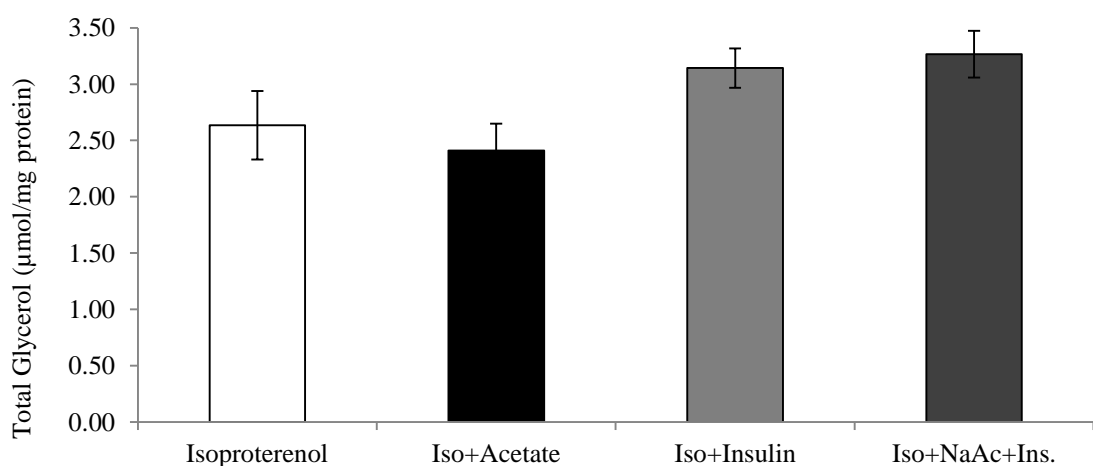


Fig. 5.5 Total glycerol was not significantly reduced in the presence of acetate. Values are mean  $\pm$  SE, n = 3 - 6.

### Adjusted concentration of glycerol in the media in the presence of isoproterenol

The effect of each treatment on the change in glycerol concentration (Fig. 5.6) did not follow the same pattern observed with NEFA (Fig. 5.2). In the presence of each treatment there was no significant reduction in the change of glycerol concentration compared with 5  $\mu$ M isoproterenol alone. Although glycerol concentration appeared to increase at each time point it was only in the Iso+Acetate treatment where glycerol concentration was observed to increase between 60 to 120 min ( $P = 0.006$ ) and from 120 to 180 min ( $P = 0.013$ ). In neither the Iso+Insulin treatment nor Iso+NaAc+Ins., did we observe any change in glycerol concentration between 60 to 120 min. However, during the final 60 min (120 -180 min) the glycerol concentration increased significantly in the presence of the Iso+Insulin ( $P = 0.053$ ) and Iso+NaAc+Ins. ( $P = 0.034$ ) treatment.

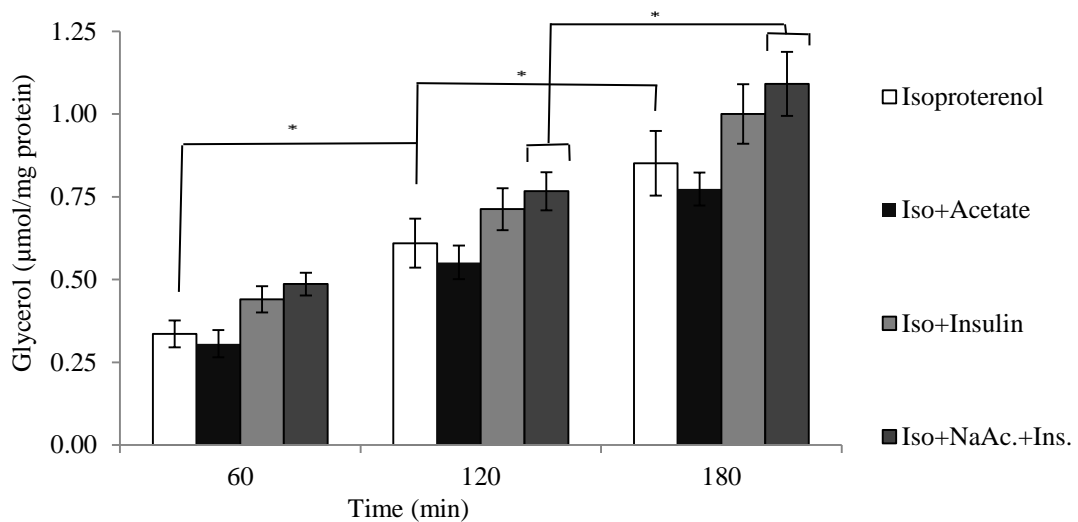


Fig. 5.6 Sodium acetate did not result in a reduction in glycerol in the presence of isoproterenol. Concentration of glycerol at 0 min was subtracted from each subsequent time point to calculate change in glycerol concentration. Values are means  $\pm$  SE, \* $P \leq 0.05$ ,  $n = 3 - 6$ .

### The effect of 4 mM sodium acetate and insulin on intracellular TAG in 3T3-L1 cultured mature adipocytes under conditions of stimulated lipolysis

Under conditions of stimulated lipolysis there was a slight decrease in the level of intracellular TAG (Fig. 5.7) compared with basal level (Fig. 4.6) as expected (300 mg/mg protein vs. 523 mg/mg protein, respectively). Iso+Acetate and Iso+Insulin did not result in a significant change in the level of intracellular TAG compared with



isoproterenol alone ( $P = 1.000$  and  $P = 0.584$ , respectively). There was also no significant change in the level of TAG in the presence of Iso+NaAc+Ins, ( $P = 0.274$ ).

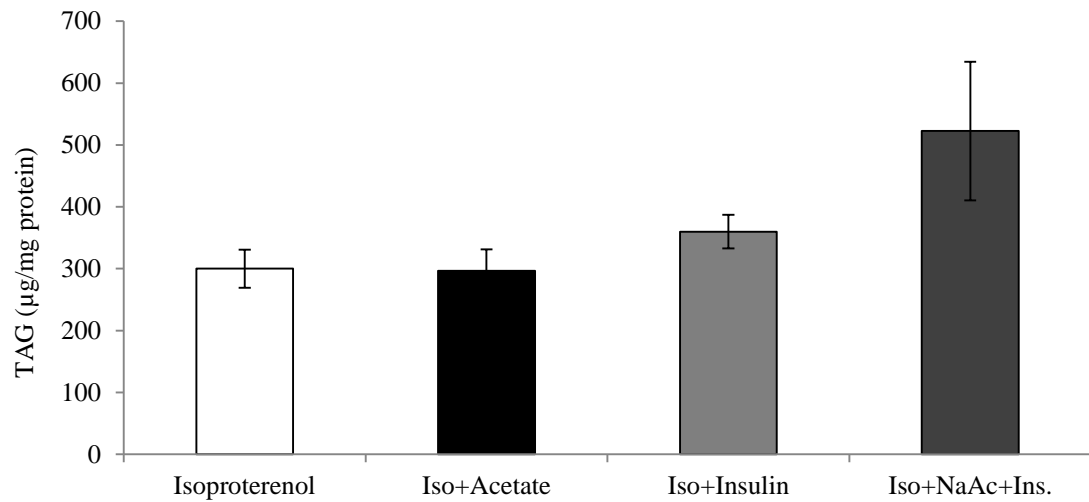


Fig 5.7 Total intracellular TAG was not significantly changed under stimulated conditions in the presence of treatment compared to control. Values are mean  $\pm$  SE,  $n = 3 - 6$ .

## Discussion

The aim of this study was to identify whether sodium acetate affects stimulated adipose tissue lipolysis *in vitro*. These data demonstrate that as hypothesised, an increase in acetate availability (4 mM) can result in a reduction in the phosphorylation of HSL at the serine 563 residue and supports the significant reduction in NEFA concentration that was observed in the media. Furthermore, as hypothesised, incubation for 120 min with 1.34  $\mu$ M insulin, reduced pHSL<sub>(SER563)</sub> by 21 % compared with isoproterenol stimulated cells alone. However, glycerol concentration did not significantly differ from controls.

The process of lipolysis is undoubtedly complex with many factors influencing this pathway; these include activation of inhibitory/stimulatory G protein-coupled receptors,  $\beta$ -adrenergic stimulation, insulin/glucagon signalling and the interactions between these factors. The regulation of lipolysis is therefore tightly controlled but also depends on the energy state of the cell, and the availability of substrates and hormones, e.g. glucose and insulin. In this study we were able to identify an intracellular change to lipid metabolism, from the basal to stimulated state, through examination of the phosphorylation status of HSL. Previously it has been shown that the phosphorylation status of HSL is critical in the catabolism of triacylglycerol. Using both an *in vitro* model and intact cell model, Fredrikson et al. (1981) demonstrated that the phosphorylation of HSL was associated with the availability of cAMP and that the enzyme activity of HSL increased two-fold in the presence of the catalytic subunit of protein kinase and cAMP. Under basal conditions of lipolysis, there was continuous low level phosphorylation of HSL<sub>(563)</sub>. Upon stimulation with the  $\beta$ -adrenergic receptor agonist isoproterenol, the level of phosphorylation increased 2.5-fold and this observation supports previous work showing an increase in pHSL in the presence of isoproterenol (Daval et al., 2005). In this study we further demonstrated that the presence of sodium acetate reduced the phosphorylation of HSL by 15 % compared with isoproterenol stimulated cells alone. To the best of our knowledge these novel findings demonstrate the ability of the SCFA, acetate, to decrease the level of phosphorylation at one of the key serine residues in HSL, at site SER563. A limitation of this study is however, the lack of data referring to the level of total HSL present in the presence of acetate treatment. This would provide a phosphorylation:non-phosphorylation ratio as another form of analysis. From the literature, it may also be of interest to investigate the effects of acetate on other phosphorylation site within HSL, including 659 and 660 (Anthonsen et al., 1998, Martin et al., 2009). Also, the effects of

acetate on the phosphorylation of serine residue 565 may be of interest as phosphorylation of this site is linked to the inhibition of HSL activity (Garton and Yeaman, 1990), and hence it may increase in phosphorylation in the presence of acetate, in a reciprocal manner to the decrease in HSL<sub>(563)</sub> observed in this study. As HSL occurs below the converging step in lipolysis, linking insulin receptor activation and GPCR activation, it would also be of interest to investigate the importance of GPR43 activation. Through the use of inhibitors of Gi coupled receptors, such as pertussis toxin, there would be more evidence for the hypothesis that acetate acts at the surface of adipocytes, and exerts its effects via a reduction in adenylate cyclase activation and therefore cAMP. Therefore, if considering the whole organism, it would also provide a novel therapeutic target for side effects of type II diabetes, including dyslipidaemia, as in this condition, insulin fails to reduce lipolysis, leading to an uncontrolled release of fatty acids and accumulation of low-density lipoprotein and very-low-density lipoprotein in the circulation (Garg and Grundy, 1990). If activation of GPR43 with supra-physiological concentrations of acetate could reduce lipolysis and hence NEFA release via an alternative pathway to insulin receptor activation, this may warrant further investigation.

As mentioned above, the most common endogenous inhibitor of the lipolytic pathway is insulin and activation of the IR and insulin receptor substrate-1 (IRS-1) is known to interact with the lipolytic pathway in adipocytes, as reviewed in detail by Duncan et al. (2007). It is well documented that under conditions of excess glucose availability, insulin not only increases glucose uptake through the GLUT4 transporter (Akiba et al., 2004) but also stimulates the PI3-Kinase pathway (Choi et al., 2010, Letiges et al., 2002). The subsequent downstream signalling process is unresolved regarding the mechanism by which insulin decreases adipocyte lipolysis, however, protein kinase B (PKB) dependent (Berggreen et al., 2009) and independent pathways (Choi et al., 2010) have been proposed to operate. Under conditions of high lipolytic stimulation, the phosphorylation of PKB on SER273 initiates the activation of phosphodiesterase 3B (PDE3B), which in turn hydrolyses cAMP into 5'-AMP (Omar et al., 2009). This conversion to 5'-AMP results in a decrease in available cAMP and therefore reduces the ability of PKA to phosphorylate HSL and perilipin, both of which are essential for hydrolysis of stored triacylglycerol (Zierath et al., 1998). Our results support these findings since in the presence of 1.34  $\mu$ M insulin; there was an observed 21 % reduction in pHSL compared with isoproterenol stimulated pHSL alone (Fig. 5.1). There was no

evidence for an additive effect in the presence of both acetate and insulin in the stimulated cells as pHSL<sub>(SER563)</sub> had only decreased by 24 % compared with isoproterenol stimulated cells. These data would suggest that both acetate and insulin are exerting their effect on the same pathway, and with a lack of an additive effect we can assume that at supra-physiological concentrations of both insulin and acetate, the pathway has reached saturation regarding the phosphorylation of HSL<sub>(SER563)</sub> .

SCFA have an affinity to GPCR, with acetate exhibiting specific affinity for GPR43 (Le Poul et al., 2003). Other studies have shown that activation of G protein-coupled receptors present in the cell membrane of adipocytes results in a reduction in the lipolytic rate as determined by the release of NEFA and/or glycerol (Koppo et al., 2012, Ge et al., 2008, Liu et al., 2009, Kong et al., 2011, Magnusson et al., 2010). These data also agree with reports showing changes in lipolysis, expressed as a reduction in the level of glycerol and/or NEFA release, in the presence of ligands of GPCR containing a small carboxylic acid tail region (Zhang et al., 2005, Taggart et al., 2005) similar to that of sodium acetate. However, the ratio between the NEFA and glycerol concentration was found to be lower than expected in this chapter. It may, however, be that re-esterification of fatty acids into the adipocyte and an increase in mitochondrial oxidation within adipocytes had taken place, which would account for the reduction in NEFA to glycerol ratio. Also, as mentioned in Chapter 4, a lack of glycerol release in the media may also be accounted for by the large background concentration also found in the treatment media. Secondly, if re-esterification is taking place, glycerol may not be released from the cell as it would be required for re-esterification. As we did not label acetate or glycerol these suggestions are only speculation, and further experimentation would be required. Finally, there appeared to be no change in the level of intracellular TAG at the end of treatment incubation. This unexpected result could be due to an error in the way the samples were extracted. After the cells were lysed, intracellular TAG had solidified around the edge of the micro-tube and therefore it was extremely difficult to include all traces of TAG for assay. In order to minimize error in future studies, methods such as organic extraction similar to that used by Miyoshi et al. (2008), and also solid phase extraction (SPE) as reviewed by Ruiz-Gutiérrez and Pérez-Camino (2000) should be considered.

In conclusion, these data support the hypothesis that increased availability of the short chain fatty acid acetate reduces the level of phosphorylation of HSL<sub>(SER563)</sub> in mature 3T3-L1 adipocytes to a level similar as that achieved in the presence of supra-

physiological concentrations of insulin. However, it would be advantageous to also consider other phosphorylation sites, and the merit of measuring total HSL. However, the decrease in pHSL(SER563) is supported by the decrease in NEFA media accumulation in the stimulated state. Glycerol accumulation in the media was not reduced to a significant extent under the same treatment conditions, however re-esterification of fatty acids and the need for intracellular glycerol could account for this lack of accumulation in the media.

## **Chapter 6**

# **Primary murine white adipocyte cell lipolysis**

## Introduction

It has been identified in Chapters 4 and 5, using the 3T3-L1 cell line, that in the presence of 4 mM sodium acetate, both basal and adrenergic stimulated lipolysis is disrupted, as shown by changes in NEFA and glycerol accumulation in the media over time. To expand on these findings, primary adipocytes were treated with acetate, insulin and NaAc+Ins. in both basal and adrenergic stimulated conditions. The results obtained would identify if primary mouse adipocytes were also susceptible to change in the presence of 4 mM sodium acetate.

Data from Chapter 4 showed that in the basal state of lipolysis in 3T3-L1 cells, acetate reduced both NEFA and glycerol after a 180 min incubation, but that these changes were not reflected in total NEFA and glycerol concentrations. Furthermore, as hypothesised, insulin did not reduce NEFA or glycerol in the basal state. Finally, acetate treatment had no effect on intracellular TAG concentration. Under  $\beta$ -AR stimulated treatment conditions in Chapter 5, however, acetate reduced total NEFA in the media from 3T3-L1 cells significantly. Therefore, it would be expected that primary murine adipocytes would be affected similarly by the same treatments. As shown by Berggreen et al. (2009), isolated rat adipocytes showed a ~ 3 fold increase in glycerol release in the presence of 100 nM isoproterenol compared with basal release, and that this was reduced by ~ 60 % in the presence of 1 nM insulin. Similarly Ge et al. (2008) noted that glycerol release from primary adipocytes isolated from epididymal fat pads increased in the presence of isoproterenol and decreased in the presence of insulin. In the same study, Ge and colleagues (2008) demonstrated a reduction in glycerol release by ~ 50 % from isolated cells incubated in the presence of propionic acid and acetic acid. These data were complementary to those observed from the 3T3-L1 cell line, under the same treatment conditions. Therefore the potential effect 4 mM acetate may have on stimulated lipolysis in isolated adipocytes may reflect those observed in previous chapters. However cell specific differences between cell line and primary cell adipocyte models (Berggreen et al., 2009), and depot specific differences within primary cell models have been observed (Caesar et al., 2010, Macotella et al., 2009).

Commonly, adipose tissue can be isolated from the subcutaneous and visceral depots (Caesar et al., 2010) of humans and mammals for use in metabolic studies. The presence of adipose tissue in locations including PG/ED and subcutaneous (SC) areas; have resulted in the identification of regional differences in adipose tissue metabolism

and function (Bolinder et al., 1983, Van Harmelen et al., 1998). As shown in a study by Macotela et al. (2009), PG/ED adipose tissue excised from female C57BL6 mice, had increased insulin sensitivity compared with adipose tissue excised from the SC region of the same animals. The sex of the animals used in experiments has also been found to change the metabolic response of adipose tissue to stimuli, as Macotela et al. (2009) demonstrated that both PG/ED and SC depots excised from C57BL6 female mice had increased insulin sensitivity compared with the same depots of C57BL6 male mice.

Therefore, from information collected from the literature, and from the results obtained in chapters 4 and 5, it was hypothesised that mature adipocytes isolated from female C57BL6 mice may be highly sensitive to insulin in the stimulated state. Similarly, as 4 mM acetate reduced NEFA to a significant extent from 3T3-L1 cells, it was hypothesised that the same concentration of acetate would also inhibit NEFA and glycerol concentration in the media from treated mature murine adipocytes after a 180 min incubation.



## Methods

All animal work was completed at the Roslin Institute, Edinburgh. An established home office licence for animal testing at the Roslin Institute covered the euthanasia process described for this study. All mice were euthanized by spinal dislocation by a trained member of staff with a personal home office licence. Fat pad isolation was also undertaken at the Roslin Institute, before isolated cells were transported back to Heriot-Watt for analysis. No ethical approval was needed for this experiment at either institute. From the literature, female C57BL6 mice have demonstrated a higher rate of adipose tissue metabolism than males and that the PG/ED adipose tissue depot is more insulin sensitive than SC depots. For this reason, the primary tissue used within this study was excised from female C57BL6 mice and isolated from the PG depot. Briefly, 6 16-week old female C57BL6/J mice were sacrificed by spinal dislocation, before the PG/ED fat pads were excised. Each set of fat pads were washed before being cut into small 2 - 4 mm pieces and placed in DB for 45 minutes, shaking at 37°C. Isolated mature adipocytes were then filtered and centrifuged for 10 min at 1100 rpm at room temperature. Isolated cells were pooled from 6 mice and split between one full panel of treatments (basal, acetate, insulin, NaAc+Ins., isoproterenol, Iso+Acetate, Iso+Insulin and Iso+NaAc+Ins.) and washed a further 2 times in KRBHA for 1 minute at 500 rpm. After the final wash step, 150 µl packed cells were plated in 600 µl plating media in a 12 well plate format. Cells were then placed at 37°C for 4 hrs to stabilize before the addition of the experimental treatments. All experiments were run in duplicate.

## Treatment

Cells that were treated with sodium acetate were spiked with 4 mM sodium acetate exactly 30 minutes prior to the start of the treatment protocol. Plating media was not aspirated at the start of the experiment, therefore at time 0, each well was spiked with a combination of stock (x100) solutions of isoproterenol (500 µM), acetate (400 mM) and insulin (134 µM) to give a final concentration of 5 µM isoproterenol, 4 mM sodium acetate and 1.34 µM insulin. Samples were collected every 60 minutes over a maximum period of 180 minutes and stored on ice. After the last sample was collected, cells were lysed, sonicated and centrifuged. Media samples were stored at -20°C and cell supernatants were stored at -80°C. Samples were analysed for NEFA, glycerol and total protein as described in Chapter 2.

## **Statistical analysis**

All statistical analysis was performed using IBM SPSS Statistics 20 software. All data sets were checked for normality using the Shapiro-Wilk test as there were < 50 samples per data set, and a boxplot was used to check for outliers. All data found to be normally distributed were analysed using either a 2-Way ANOVA with post-hoc Tukey test or a 1-Way ANOVA with post-hoc Tukey test. All paired non-parametric data were analysed with Friedmans, and independent non-parametric data was analysed using the Kruskal-Wallis with pair-wise Mann-Whitney. A  $P$  value of  $\leq 0.05$  was considered statistically significant.

## Results

### The effect of 4 mM sodium acetate and insulin on basal NEFA in the media from mature murine adipocytes

As shown in Fig. 6.1, there were no significant changes in NEFA in the media in the presence of acetate, insulin or NaAc+Ins. compared with basal treatment at 0 min ( $P = 0.578$ ,  $P = 0.999$  and  $P = 0.987$  vs. basal, respectively); 60 min ( $P = 0.704$ ,  $P = 0.997$  and  $P = 1.000$  vs. basal, respectively); 120 min ( $P = 0.590$ ,  $P = 0.970$  and  $P = 0.985$  vs. basal, respectively) or 180 min ( $P = 0.775$ ,  $P = 0.992$  and  $P = 1.000$  vs. basal, respectively). NEFA concentration did not increase over time, as the concentration of NEFA in the media at time 0 min (basal  $0.043 \pm 0.007$   $\mu\text{mol}/\text{mg}$  protein; acetate  $0.058 \pm 0.003$   $\mu\text{mol}/\text{mg}$  protein; insulin  $0.045 \pm 0.002$   $\mu\text{mol}/\text{mg}$  protein; NaAc+Ins.  $0.046 \pm 0.014$   $\mu\text{mol}/\text{mg}$  protein) was not significantly different from the concentration of NEFA in the media at 180 min (basal  $0.037 \pm 0.005$   $\mu\text{mol}/\text{mg}$  protein; acetate  $0.046 \pm 0.004$   $\mu\text{mol}/\text{mg}$  protein; insulin  $0.040 \pm 0.000$   $\mu\text{mol}/\text{mg}$  protein; NaAc+Ins.  $0.036 \pm 0.012$   $\mu\text{mol}/\text{mg}$  protein)  $P = 0.397$ .

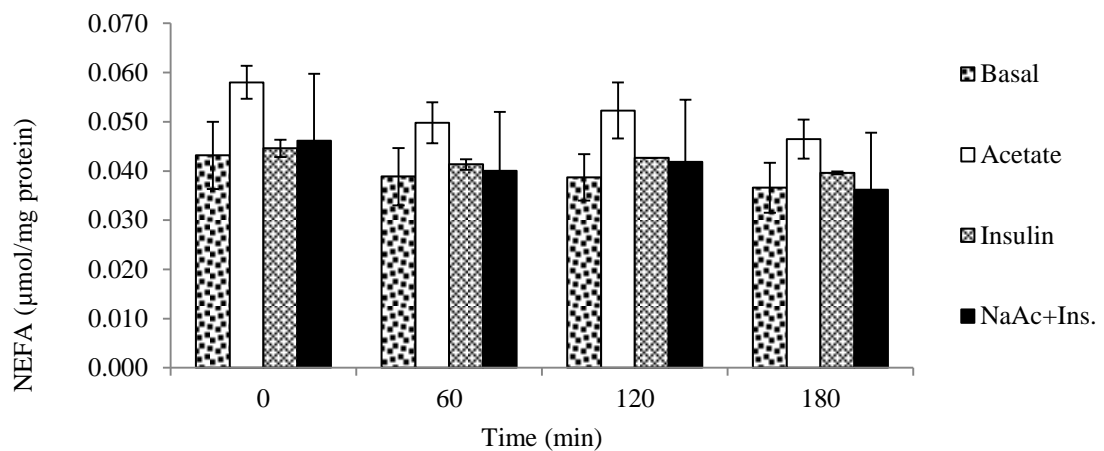


Fig. 6.1 Acetate did not result in a significant change in NEFA at any time point over the 180 min incubation compared with the basal state. Values are mean  $\pm$  SE.  $n = 2$ .

### Total basal NEFA concentration over 180 minutes

The total accumulation of NEFA in the media over the 180 min incubation did not change significantly between the 4 treatment groups ( $P = 0.771$ ), as shown in Fig. 6.2. Compared with basal treatment alone, acetate, insulin and NaAc+Ins. treatment did not

results in a significant reduction in NEFA ( $P = 0.838$ ,  $P = 0.999$  and  $P = 0.998$  vs. basal, respectively).

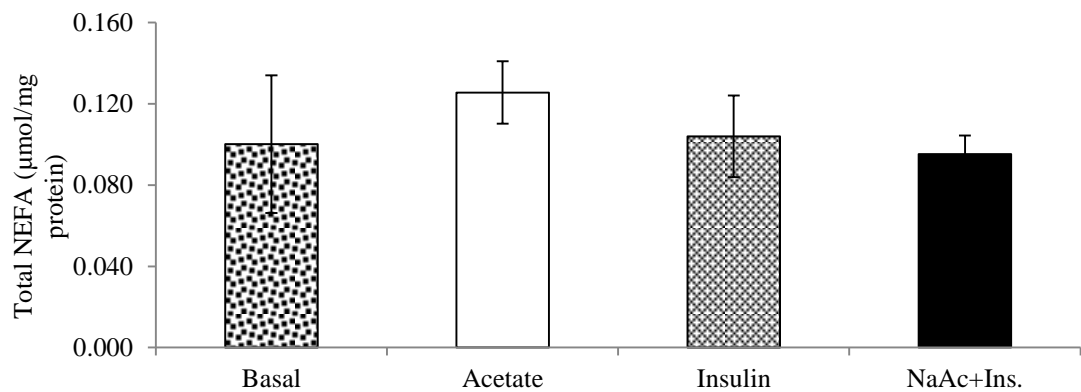


Fig. 6.2 Total NEFA did not significantly change in the presence of acetate, insulin or NaAc+Ins. compared to basal treatment. The total concentration of NEFA in the media is expressed at mean  $\pm$  SE.  $n = 2$ .

#### The effect of 4 mM sodium acetate and insulin on basal glycerol in the media from mature murine adipocytes

As shown in Fig. 6.3, in the basal state neither acetate, insulin or NaAc+Ins. resulted in a significant change in glycerol at 0 min ( $P = 0.359$ ), 60 min ( $P = 0.563$ ), 120 min ( $P = 0.823$ ) or 180 min ( $P = 0.710$ ). Similarly, there was no significant change in glycerol concentration between time points (0 – 60,  $P = 0.999$ ; 60 – 120,  $P = 0.995$  and 120 – 180,  $P = 0.565$ ). When comparing glycerol concentrations it was also apparent that glycerol concentration in the media after all treatments at 180 min, was not significantly different than glycerol concentration at 0 min ( $P = 0.609$ ).

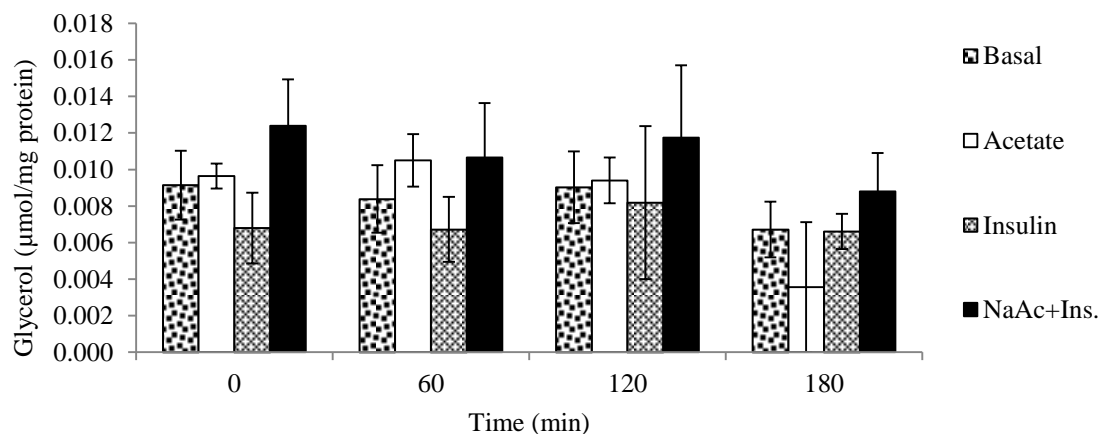


Fig. 6.3 In the basal state, the presence of acetate did not result in a significant change in glycerol over 180 min incubation. Values are mean  $\pm$  SE.  $n = 1 - 2$ .

### Total basal glycerol concentration over 180 minutes

The total concentration of glycerol in the media in the basal state was  $0.02 \pm 0.01$   $\mu\text{mol}/\text{mg}$  protein over the 180 min incubation, as shown in Fig. 6.4. Total glycerol in the presence of acetate, insulin and NaAc+Ins. did not significantly differ from glycerol in the basal state ( $P = 1.000$ ;  $P = 0.997$  and  $P = 0.969$  vs. basal, respectively).

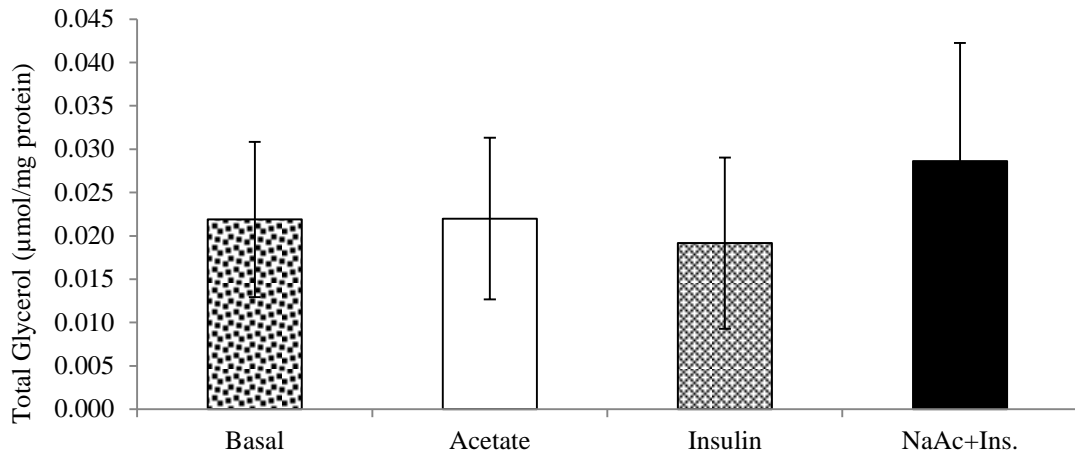


Fig. 6.4 Total glycerol in the presence of acetate did not significantly change compared to basal state. The total concentration of glycerol in the media is expressed at mean  $\pm$  SE.  $n = 2$ .

### The effect of 4 mM sodium acetate and insulin on stimulated NEFA in the media from mature murine adipocytes

As shown in Fig. 6.5, in the presence of  $5 \mu\text{M}$  isoproterenol, NEFA did not increase significantly between 0 min ( $0.044 \pm 0.002$   $\mu\text{mol}/\text{mg}$  protein) and 180 min  $0.042 \pm 0.002$  ( $P = 0.981$ ). Also, treatment of primary mature adipocytes with Iso+Acetate did not result in a significant change in NEFA concentration in the media compared with isoproterenol alone at any time point over the 180 min incubation (0 min  $P = 0.896$ , 60 min  $P = 0.823$ , 120 min  $P = 0.297$  and 180 min  $P = 0.114$ ). A comparison of Iso+NaAc+Ins. with isoproterenol alone was conducted on NEFA over the 180 min incubation, and resulted in a significant change in NEFA concentration ( $P = 0.033$ ). However, after further pairwise comparisons at each time point (0, 60, 120 and 180 min), the effect of Iso+NaAc+Ins. was not statistically significant from isoproterenol alone ( $P = 0.711$ ,  $P = 0.507$ ,  $P = 0.421$  and  $P = 0.090$  vs. isoproterenol, respectively).

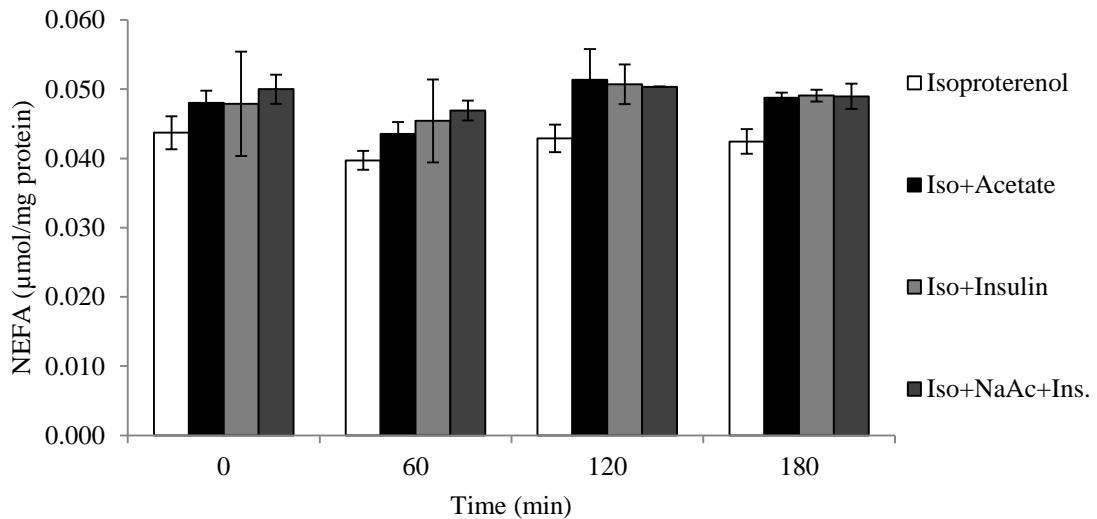


Fig. 6.5 In stimulated conditions, acetate did not result in a significant change in NEFA concentration compared to control. Values are mean  $\pm$  SE.  $n = 2$ .

#### Total stimulated NEFA concentration over 180 minutes

The total accumulation of NEFA in the media under stimulated conditions did not differ significantly between treatment groups ( $P = 0.343$ ), as shown in Fig. 6.6. Compared with isoproterenol alone, Iso+Acetate, Iso+Insulin and Iso+NaAc+Ins. did not result in a significant reduction in NEFA ( $P = 0.475$ ,  $P = 0.421$  and  $P = 0.372$ , respectively).

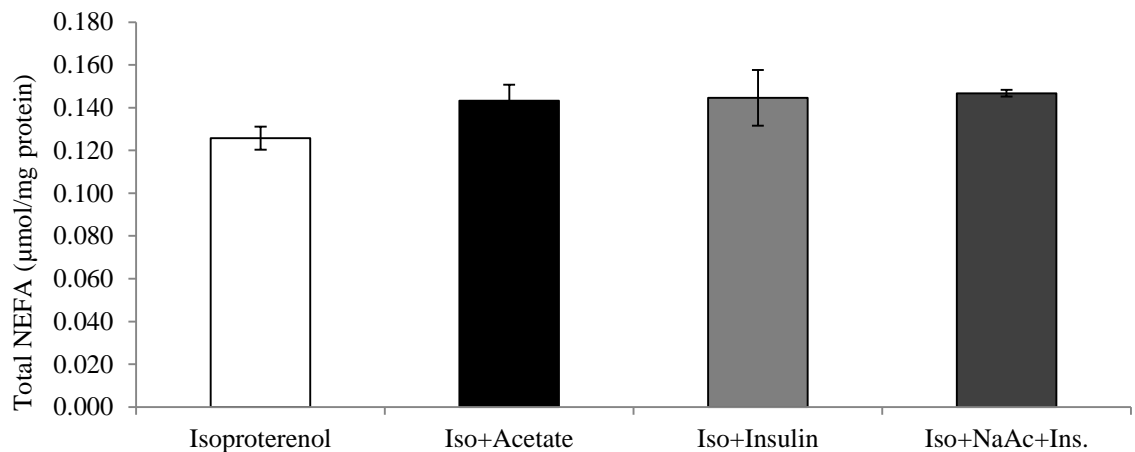


Fig. 6.6 Total NEFA concentration in the media in  $\beta$ -AR stimulated conditions did not change significantly in the presence of acetate, insulin or NaAc+Ins. after a 180 min incubation. The total concentration of NEFA is expressed as mean  $\pm$  SE.  $n = 2$ .

### The effect of 4 mM sodium acetate and insulin on stimulated glycerol in the media from mature murine adipocytes

As shown in Fig. 6.7, in the presence of  $\beta$ -AR stimulation, glycerol did not change significantly over the 180 min incubation period ( $P = 0.102$ ). Similarly, stimulated cells treated with Iso+Acetate, Iso+Insulin and Iso+NaAc+Ins. did not result in a change in glycerol over the 180 min incubation ( $P = 0.257$ ,  $P = 0.414$  and  $P = 0.157$ , respectively). There was no effect of treatment conditions on glycerol concentration in the media compared with stimulated treatment alone. As expected there was no significant difference between treatment conditions Iso+Acetate, Iso+Insulin and Iso+NaAc+Ins. at time 0 min vs. isoproterenol ( $P = 0.480$ ,  $P = 1.000$  and  $P = 1.000$ , respectively). In addition, there was also no difference observed at 60 min ( $P = 0.221$ ;  $P = 0.683$  and  $P = 0.683$ ), 120 min ( $P = 0.157$ ,  $P = 0.317$  and  $P = 0.480$ ) or at 180 min ( $P = 1.000$ ,  $P = 0.157$  and  $P = 1.000$ ).

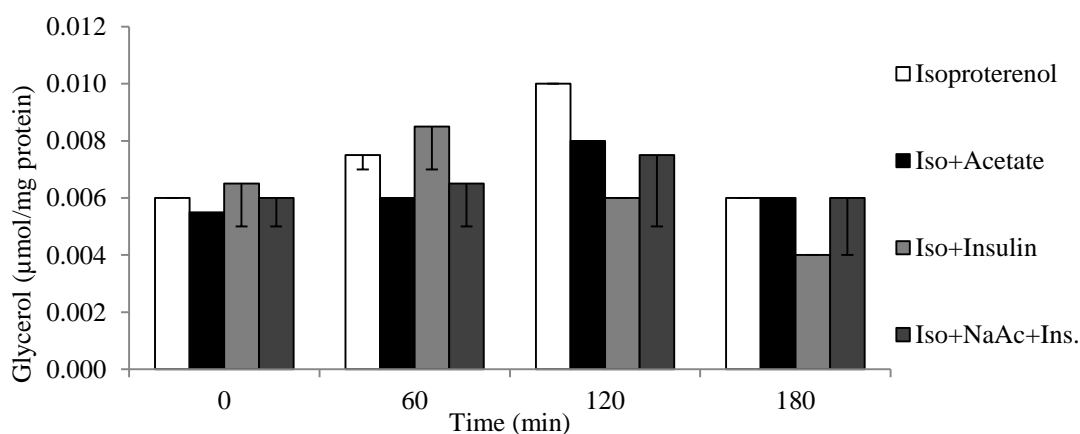


Fig. 6.7 Under  $\beta$ -AR stimulation, acetate did not result in a significant change in glycerol within the 180 min incubation. Data expressed as median and quartiles.  $n = 1 - 2$ .

### Total stimulated glycerol concentration over 180 minutes

Total concentration in the media of glycerol under isoproterenol stimulation over 180 min ( $0.027 \pm 0.003$   $\mu\text{mol/mg protein}$ ) was not significantly different from total glycerol in the presence of Iso+Acetate  $0.020 \pm 0.002$   $\mu\text{mol/mg protein}$ ; Iso+Insulin  $0.026 \pm 0.008$   $\mu\text{mol/mg protein}$  and Iso+NaAc+Ins.  $0.020 \pm 0.001$   $\mu\text{mol/mg protein}$ , ( $P = 0.841$ ,  $P = 0.999$  and  $P = 0.810$  respectively), as shown in Fig. 6.8.

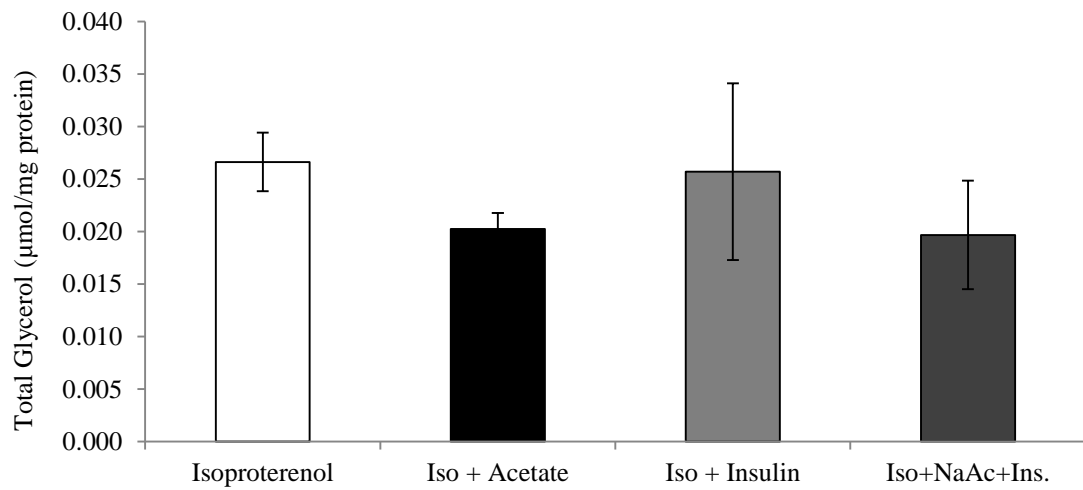


Fig. 6.8 Total glycerol in the media under stimulated conditions was not significantly different in the presence of any treatment condition compared with control. The total concentration of glycerol is expressed as mean  $\pm$  SE. n = 2.



## Discussion

From data collected in this study, treatments containing acetate, insulin and NaAc+Ins. did not result in a change of NEFA and glycerol in either the basal or  $\beta$ -AR stimulated state. There appeared to be no concentration change in NEFA under basal conditions and limited change in stimulated conditions compared with time 0 min. Glycerol accumulation in the media over 180 min, and total glycerol in the media after a 180 min incubation was not significantly changed in the basal or stimulated state in the presence of 4 mM acetate. Also, insulin did not significantly reduce NEFA or glycerol in the basal or stimulated state over the 180 min incubation, and total accumulation in the media in the presence of insulin was unaffected compared with controls.

Primary white adipocyte cell isolation is complex in that it covers the isolation of mature adipocytes and/or the stromal vascular cell (SVC) fraction for pre-adipocyte differentiation (Fernyhough et al., 2004). Differences in DB, filter size and culture media exist between these protocols (Fernyhough et al., 2004). There is even evidence to suggest standard isolation protocols can result in physiological changes to cell function, including activation of inflammatory responses (Ruan et al., 2003). The method chosen for these experiments utilised a combination of both typical adipocyte collagenase and liberase, which contained a blend of both collagenase type I and type II. When considering the reliability of primary cell experiments, the viability of the isolated cell population is very important. Identification of DB3 in preliminary experiments (Fig. 3.20), was used as it had demonstrated a mix of both collagenase and liberase was most effective at isolating the most viable cells. It was therefore surprising to find that over the course of the 180 incubation, NEFA concentration in the basal state remained negligible (Fig. 6.1). In comparison with Taggart et al. (2005), who reported a release of 0.25  $\mu\text{mol/hr}$  NEFA in the presence of 1  $\mu\text{M}$  isoproterenol, the data presented in Fig. 6.5 show the average concentration of NEFA to be 0.042  $\mu\text{mol/hr}$ , a  $\sim 6$  fold decrease in concentration. The background level of NEFA present in the media at 0 min was also  $\sim 2$  fold higher than represented in the paper by Taggart et al. (2005), however this may be due to differences in protocol used in this thesis. Isolated cells were left to stabilize for 4 hrs prior to the start of the experiment. Cell media was then spiked with stock treatments, leading to the high background concentrations of NEFA and glycerol present in the time 0 min samples. Comparisons with the literature have shown data from Chapter 6 do not fit well with expected results, however, it is of interest to identify if these findings complement cell line data from Chapter 4 and 5.

In this study, the concentration of total NEFA accumulation in the media from primary murine white adipocytes (Fig. 6.2) was roughly 4 fold lower than that of 3T3-L1 cell in the presence of basal and NaAc+Ins. treatments (Fig 5.3). Similarly, in the basal state, total NEFA in the media from mature primary white adipocytes treated with acetate for 180 min was roughly 2.5 fold lower (Fig 6.2) than total NEFA accumulation in the media from acetate treated 3T3-L1 cells (Fig 5.3). In addition, treatment with insulin in the basal state resulted in a 5-fold difference in total NEFA accumulation in the media from primary murine white adipocytes compared with 3T3-L1 cells (Fig 5.2 and Fig 6.3, respectively). As shown by Taggart et al. (2005), data collected on the isolated epididymal adipocytes of wild type C57BL6 mice, NEFA release increased significantly under  $\beta$ -AR stimulation with isoproterenol. This increase in NEFA was absent from the data collected within this thesis, as shown by a lack of change in NEFA concentration between basal (Fig 6.1) and isoproterenol (Fig. 6.5) stimulated cells. Similarly, when comparing total glycerol in the media from primary murine white adipocytes with 3T3-L1 cell line under basal and stimulated treatments, the 3T3-L1 cell line resulted in a 100 fold higher concentration in total glycerol after  $\beta$ -AR stimulation, whereas no change was observed from primary cells. Furthermore, in the stimulated state, there was no significant change between treatments in the primary murine white adipocyte study compared to isoproterenol treatment alone. From the data, it can be concluded that the primary tissue glycerol concentration is below expected values, as compared with other primary glycerol release concentrations (Qiao et al., 2011).

Due to the results observed in this chapter, cell viability may be questioned when comparing basal to stimulated primary cells. Also, the effect of pooling adipocytes from 6 different animals may also be a limiting factor. As stated in the methods section, it was not possible to rule out the presence of other cells, such as macrophages, within the isolated adipocyte layer after washing and filtration steps. Consideration should therefore be given to possible effects from combining cells from more than one animal, however as this is only speculation, this would need to be investigated further.

As shown in Fig. 6.4, total glycerol accumulation in the media in the basal state ( $0.02 \pm 0.01$   $\mu\text{g}/\text{mg}$  protein) was similar to the concentration of glycerol present in the isoproterenol stimulated cells, shown in Fig 6.8 ( $0.02 \pm 0.01$   $\mu\text{g}/\text{mg}$  protein) after a 180 min incubation. Experiments using primary mouse adipocytes conducted by Qiao et al. (2011), illustrated that in wild type animals, serum glycerol increased from  $\sim 0.2$   $\mu\text{mol}/\text{mg}$  tissue to  $\sim 1.8$   $\mu\text{mol}/\text{mg}$  tissue in the presence of the  $\beta_3$ -Adrenergic receptor

agonist BRL37344. This would lead us to believe that cells in the study described in Chapter 6 in both basal and stimulated cell groups were not viable. This could have been confirmed had a cytotoxicity assay been performed at the end of the experiment to address if cells were still metabolising normally.

In conclusion, studies looking at the metabolic function of mature isolated adipocytes have shown that under  $\beta$ -AR stimulation, both NEFA and glycerol release increases over time compared to the basal state (Taggart et al., 2005, Qiao et al., 2011). However, within Chapter 6, results have shown no such increase in either media NEFA or glycerol during a 180 min incubation. Similarly, the presence of acetate and insulin treatments did not result in a similar decrease in NEFA and glycerol from primary mature white adipocytes compared with data presented in previous chapters on 3T3-L1 cell metabolism. Cell cytotoxicity was not assessed using the resazurin technique on these cells, and the effect of combining cells from more than one animal were also not investigated, however, from data collected on NEFA concentration from both basal and stimulated cells, it would appear that for the duration of the 180 min incubation, isolated mature adipocytes were not metabolising as evidenced by a lack of NEFA and glycerol accumulation into the culture media.

# **Chapter 7**

## **General Discussion**

## Overview

This thesis has aimed to explore the effects of increasing dietary available compounds, such as SCFA, to levels above that found naturally in the circulation, on adipose tissue metabolism and function *in vitro*. Previously, supra-physiological concentrations of compounds have been identified as having therapeutic properties, including, nicotinic acid/niacin as a treatment for cardiovascular disease, which exploits the activation of HM74A, but at higher levels than normally found in humans (Pike, 2005, Zhang et al., 2005). Dietary intake of carbohydrates, alcohol consumption and level of oxidation within tissues all contribute to changes in the concentration of acetate available in plasma, however, it is concentrations above those found naturally which may provide evidence for a therapeutic target in the prevention of a side effect of type II diabetes, dyslipidaemia. As described in previous chapters, endogenous levels of acetate rarely increase above 2 mM, however artificially increasing the level of acetate, and the subsequent reduction of pHSL and NEFA concentration shown in this thesis, that is of interest. These results will be discussed in relation to GPCR and insulin signalling, regarding a potential mechanism of action within adipocytes. The discussion will compare results from this thesis with published studies, to identify similarities and differences in the data, so that changes observed in 3T3-L1 adipocyte in the presence of increased SCFA can be better understood.

### Short chain fatty acid acetate availability

Previous publications have shown that intake of certain food groups influence the gut microbiota, and in turn affect the production of certain dietary available compounds, including the appearance of SCFA (Karlsson et al., 2013), through intake of non-digestible carbohydrates and metabolism of alcohol in the liver (Macfarlane and Macfarlane, 2003, Al-Lahham et al., 2010, Siler et al., 1999, Puupponen-Pimiä et al., 2002). Although measurement of exogenous concentrations of SCFA produced by gut fermentation were conducted (Macfarlane and Macfarlane, 2003), and methods for crossing the gut barrier were proposed (McNeil et al., 1978), measuring the level of endogenous concentrations of SCFA were not easily accessible as methods used to measure portal and hepatic SCFA concentrations were considered highly invasive. However, since the first measurement of portal blood SCFA by Peters et al. (1992), and subsequent *in vivo* human experiments, there are data to suggest, both propionate and butyrate are utilised for gut membrane health or metabolised quickly in the liver after

entering the circulation (Bloemen et al., 2009). Furthermore, the concentration of acetate found in the circulation is much larger than that of propionate and butyrate (Siler et al., 1999). Identification of increased acetate availability *in vivo*, has led to studies that show acetate to be oxidised within the brain (Volkow et al., 2013, Jiang et al., 2013) and heart (Yamashita et al., 2001), as well as the presence of SCFA receptors (Sawzdargo et al., 1997) within adipose tissue (Ge et al., 2008), and also the large intestine and islets of mice (Leonard and Hakak, 2006). Concentrations of between 1 - 2 mM acetate have been observed in plasma after infusion of 350 mM acetate in human studies (Jiang et al., 2013) and in the hepatic vein after controlled ingestion of ethanol (Nuutinen et al., 1985). As shown in Fig. 3.7, initial studies to identify the active concentration of acetate on adipocyte lipolysis demonstrated that a concentrations of 2 mM was able to reduce NEFA in the media after 180 min incubation, but had no significant effect on glycerol. A concentration of 4 mM was required to reduce NEFA accumulation in the media after 120 min incubation in day 9 post-differentiated 3T3-L1 cells. Sodium acetate appears to be selective for a G protein-coupled receptor GPR43 (Ge et al., 2008). There are also data to suggest artificially increased concentrations of acetate, are sufficient to reduce lipolysis *in vivo* (Smith et al., 2007). Nilsson and Belfrage (1978) noted that primary isolated rat adipocytes exhibited a dose dependant reduction in glycerol release, by 10 %, in the presence of 2 mM acetate and by 25 - 70 % in the presence of 10 mM acetate. In Chapter 5, in the presence of 5  $\mu$ M isoproterenol, 4 mM acetate reduced NEFA from day 9 post-differentiated 3T3-L1 cells by ~35 % after a 180 min incubation, compared to isoproterenol stimulated cells alone (Fig. 5.2). Therefore, previous studies have identified a GPCR specific for SCFA within adipose tissue, and that the presence of SCFA can result in a reduction in release of the products of lipolysis, including NEFA and glycerol, comparable with data presented in this thesis. However, questions remained regarding the intracellular mechanism(s) linking extracellular surface receptor activation with NEFA and glycerol release. Utilising techniques such as western blotting for markers of lipolysis, including HSL would be one way of addressing this issue.

### **GPCR and insulin receptor activation as a regulator of adipocyte lipolysis**

The lipid stored within adipocyte droplets is in a state of constant turnover, regulated in part, by the activation of surface receptor agonists and hormone sensing, as reviewed by Fain and García-Sáinz (1983), Carmen and Víctor (2006). Both stimulatory and

inhibitory agonists act simultaneously to maintain the homeostatic balance of TAG hydrolysis and re-esterification. In the basal state when cells are not predominantly activated by stimulatory compounds such as catecholamines, the concentration of NEFA and glycerol is lower than that observed in stimulated cells as expected (Table 5.1 and Table 5.2, respectively). Activation of the lipolytic pathway increases as a result of  $\beta$ -AR activation. When glucose is in short supply and the body requires an increase in ATP availability for energy, an increase in lipid metabolism is required to hydrolyse free fatty acids for use in mitochondria. This increase has been known to occur in response to fasting, acute stress (Weissman, 1990) and smoking (Andersson and Arner, 2001). Signals can originate extracellularly from areas including the CNS, intestinal tract and pancreas (Lustig, 2008). As shown in Fig. 3.5, the dose response relationship for isoproterenol on NEFA accumulation in the media was not linear. The concentration required to activate 100 % NEFA accumulation in the media was  $\sim 10 \mu\text{M}$ , complementary to concentrations used in other studies (Greenberg et al., 2001 {Sztalryd, 2003 #200, Brasaemle et al., 2004}). As the data suggest in Fig. 3.6, 4 mM acetate was unable to exert any reduction in NEFA media accumulation, therefore a sub-maximal concentration of  $5 \mu\text{M}$  was chosen.. Previously, Tavernier et al. (2005) reported an isoproterenol  $\text{EC}_{50}$  on glycerol release of  $462 \pm 146 \text{ nm}$  in adipocytes isolated from wild type male rodents, with a 14 fold increase to  $6246 \pm 5572 \text{ nm}$  in isolated adipocytes from  $\beta$ -AR knockdown animals ( $\beta 1^{-/-}/\beta 2^{-/-}/\beta 3^{-/-}$ ). However, as shown in Fig. 3.6 of this thesis, the concentration required to maximally stimulate accumulation of glycerol from mature 3T3-L1 cells was  $\sim 10 \mu\text{M}$  isoproterenol. Differences in the sensitivity of adipocytes to isoproterenol may have occurred due to the use of primary cell and cell line models, and also the volume of tissue used. For this reason, an  $\text{EC}_{50}$  concentration of  $5 \mu\text{M}$  isoproterenol was used throughout this thesis.

Studies show a range of receptors present in the adipocyte cell membrane, each of which contributes to the complexity of regulating the lipolytic/lipogenic relationship (Castan et al., 1994, Hong et al., 2005, Ge et al., 2008, Zhang et al., 2005, Zierath et al., 1998). As discussed in Chapter 1, there are many surface receptors which are Gi protein-coupled, and are capable of lipolytic inhibition, including GPR43 and HM74A. Activation of the EP3 prostaglandin receptor by  $\text{PGE}_2$  has also been reported to result in inhibition of lipolysis in mature adipocytes (Castan et al., 1994, Jaworski et al., 2009). There are also multiple compounds that activate these receptors including niacin, ketone bodies and short chain fatty acids (Nilsson and Belfrage, 1978, Pike, 2005, Taggart et

al., 2005). A similarity between the agonists that activate the GPCR Gi may warrant further investigation, as each receptor agonist contains a small carboxylic acid tail region (Chang et al., 1997). Acetate is a two carbon molecule with a single carboxyl group, and this tail region is shared by endogenous and artificial agonists, including niacin for HM74A (Pike, 2005, Zhang et al., 2005) and PGE<sub>1</sub> for EP3 receptors (Green and Johnson, 1991). However, the most common endogenous lipolytic inhibitor insulin acts via the insulin receptors present on adipocyte cell surface. The action of insulin to suppress lipolysis (Zierath et al., 1998), is secondary to increasing glucose uptake via Glut4 transporters and increasing adipogenesis as reviewed by Wilcox (2005).

Activation of the insulin pathway has been well documented in adipose tissue (Koppo et al., 2012, Duncan et al., 2007, Ariotti et al., 2012, Berggreen et al., 2009). An increase in extracellular glucose availability not only results in an increase in the release of insulin from pancreatic  $\beta$ -cells, but also an increase in Glut4 translocation to plasma membrane of adipose tissue (Sano et al., 2011). Insulin binds to the insulin receptors present in the cell membrane of adipocytes, resulting in a decrease in lipolysis. Although the mechanism of action has been disputed in recent years (Choi et al., 2010), there is a general consensus that the activation of PDE3B converts cAMP into 5'AMP, and eventually decreases TAG hydrolysis and therefore NEFA and glycerol release (Berggreen et al., 2009, Kitamura et al., 1999). As described in Chapter 2, human recombinant insulin was used throughout these studies at a concentration of 1.34  $\mu$ M. However, as shown in Fig. 4.1 and Fig. 4.3, and Fig. 5.2 and Fig 5.4; at no point did 1.34  $\mu$ M insulin alone result in a decrease in NEFA and/or glycerol accumulation in the media from mature 3T3-L1 cells. The proposed concentration of insulin used in these studies would be regarded as high, as others have used a much lower concentration and still observed inhibition or reduction in lipolysis (Cheatham et al., 1994). A study by Mulder et al. (2005) on insulin induced Glut4 transport used 0.001 - 1  $\mu$ M insulin. They identified that 2-deoxyglucose uptake in the presence of 1  $\mu$ M insulin was reduced in the presence of the  $\beta$ -AR receptor agonist, epinephrine, compared with insulin alone. Bolinder et al. (1983) used a range of insulin from 1 - 1000  $\mu$ U/ml to detect changes in glycerol release as a measure of lipolysis in different adipose depots. They identified subcutaneous adipocytes were more sensitive to a change in insulin concentration than omental depots. Data gathered from metabolic studies within this thesis have demonstrated that insulin at a concentration of 1.34  $\mu$ M was unable to reduce NEFA and/or glycerol accumulation in the media. However, when investigating HSL levels in



treated cells, under stimulated conditions, insulin alone reduced phosphorylation of HSL<sub>SER563</sub> by 21 %. Under post prandial conditions, in humans, insulin levels only reach a concentration of ~350 pM (Poretsky, 2010), a quarter less than used in this study, and so 1.35  $\mu$ M would be considered supra-physiological.

There is a number of alternative phosphorylation sites to SER563, including SER565, SER600, SER659 and SER660 (Anthonsen et al., 1998, Greenberg et al., 2001, Martin et al., 2009). These sites may also be affected in the presence of increased acetate availability. From Fig. 5.1, pHSL<sub>SER563</sub> increased by 2.5 fold in the presence of 5  $\mu$ M isoproterenol, compared to the basal state. A study in 2001 showed that catecholamines not only stimulated  $\beta$ 3-activation but also activated extracellular signal-regulated kinase (ERK), controlling a novel HSL phosphorylation site at SER600 to increase lipolysis (Greenberg et al., 2001). The increase in pHSL<sub>SER563</sub> observed in the presence of half maximal  $\beta$ -AR stimulation by isoproterenol, was reduced by 21 % in the presence of the endogenous lipolytic inhibitor insulin, compared with stimulated cells alone. Also, in the stimulated state, it was shown that phosphorylation of HSL<sub>SER563</sub> was reduced by 15 % in the presence of 4 mM acetate compared to stimulated pHSL alone. It would therefore be of interest to investigate the effect of increased acetate availability on the phosphorylation of HSL<sub>SER565</sub>, as it was identified that phosphorylation of SER565 occurs under basal conditions, in the absence of lipolytic stimulation (Garton and Yeaman, 1990). Similarly, it was shown in a study by Martin et al. (2009), that phosphorylation of the HSL<sub>SER565</sub> residue was inversely proportional to the phosphorylation of HSL<sub>SER563</sub> (Greenberg et al., 2001). Identification of the importance of SER659 and SER660 to the activation of HSL should also be considered, as Anthonsen et al. (1998) showed that mutation of both SER659 and SER660, completely abolished HSL activation in COS cells. However, mutation of only one serine site did not result in the same level of reduced activation compared to the mutation of both SER659/660. It is also of interest to mention the limitations of these studies. As mentioned in previous chapters, due to a lack of data on total HSL levels present in the stimulated state in the presence of 4 mM acetate, we cannot provide a phosphorylation:non-phosphorylation ratio, which would define the exact level of pHSL(SER563) inhibition. However, complementary to these data, it was also identified that NEFA in the basal and stimulated state was also reduced in media in the presence of 4 mM acetate, however unlike our original hypothesis, under stimulated conditions; glycerol was not affected, compared with controls. Again, a limitation of the

data presented in this thesis should be considered when referring to accumulation of NEFA and glycerol in the media, which cannot be confused with measures of absolute release. As re-uptake of NEFA and glycerol were not calculated, via labelling techniques, absolute release cannot be calculated. In support of this, evidence of re-uptake is apparent in the basal state, where there is less accumulation of NEFA in the media after 180 min compared with accumulation at 120. Similarly, a lack of change in glycerol release over time, may suggest glycerol is being re-esterified with available fatty acids, which could include the test compound acetate. Although this cannot be determined from the data presented in this thesis, acetate is not accounted for in the NEFA assay as evidenced by a lack of NEFA at time 0 min, showing a specificity for LCFA and MCFA.

### **Increased acetate availability on NEFA, glycerol and TAG concentrations**

Overall, it can be concluded from the data presented in the previous chapters (4 and 5), that excess sodium acetate availability has a small but significant inhibitory effect on both basal and stimulated lipolysis of white adipose 3T3-L1 cells. The basal rate of lipolysis was reduced in the presence of 4 mM acetate as shown by a reduction in both NEFA and glycerol in the media after a 180 min incubation, however intracellular TAG concentration remained unchanged compared with controls. Data published by Ge et al. (2008), demonstrated basal plasma FFA from C57BL6 mice was reduced in the presence of 500 mg/kg acetate in less than 30 min and that % glycerol release was also reduced in the presence of sodium acetate compared to vehicle. However, calculation of total NEFA and total glycerol in the basal state, in the presence of acetate, insulin and NaAc+Ins., showed there to be no overall reduction compared to controls, similar to data published by Nilsson and Belfrage (1978) and Morimoto et al. (1998). In the presence of  $\beta$ -AR stimulated lipolysis, acetate significantly reduced NEFA in the media; however, both glycerol and TAG concentration remained unchanged compared with controls. Previously, Zhang et al. (2005) reported a reduction in forskolin-induced intracellular cAMP levels and decreased glycerol release after activation of GPCR HM74A with niacin, in 3T3-L1 cells. Smith et al. (2007) also demonstrated that 60 min after ingestion of a sodium acetate bolus, a plasma acetate concentration of ~1.3 mM could result in a decrease in plasma FFA by 19 % compared with time 0. Similarly, as shown in Fig. 5.2, NEFA concentration in the media was reduced by 22 % compared with control in the presence of Iso+Acetate, after 60 min of incubation. Therefore, data

presented in Chapter 5 complement data from published studies utilising SCFA and GPCR agonists regarding their effect on adipose tissue lipolysis. However, as noted previously, in contrast to published data, total glycerol concentration measured in the presence of each treatment did not reduce compared to controls.

Previously, studies have shown evidence of re-esterification of fatty acids within adipocytes, where NEFA is re-esterified into TAG for storage in lipid droplets (Rodrigues et al., 2013, Zimmermann et al., 2003). It is therefore possible that acetate not only acts to decrease pHSLSER563, but may also increase NEFA re-esterification and lipogenesis. Data presented in Fig. 5.2 and Fig. 5.4, show that after 180 min incubation, the ratio of glycerol to NEFA was not 3:1 as expected. Instead, a ratio of 1.5:1 was observed in this study under stimulated conditions. Complementary to these data, in the basal state, after 180 min incubation, there was a negative concentration of NEFA, where the concentration of NEFA in the media was lower at 180 min compared with 120 min. This would suggest that NEFA was taken back into the cell to be utilised. Unfortunately, the method of TAG analysis used in this study was not accurate enough to clarify if NEFA was oxidised within the cell or converted back into TAG within lipid droplets. Other methods may have been more reliable for TAG analysis and NEFA incorporation (Miyoshi et al., 2008). From data gathered within these studies, it would therefore be of interest to investigate if similar changes were observed in isolated primary mature adipocytes.

The total NEFA in the media from primary murine white adipocytes in the basal state, was found to be 4 fold lower compared with that from the 3T3-L1 cell line in two treatment conditions, basal and NaAc+Ins. Concentrations of total NEFA in the media from primary cells in the remaining treatment conditions, acetate and insulin, were ~2.5 fold and ~5 fold lower, respectively compared with the 3T3-L1 cell line. Similarly, this pattern was also shown in the stimulated studies as the difference in total NEFA accumulation in the media in all treatment conditions between cell line and isolated primary adipocyte cells was over 14 fold. Total glycerol from the 3T3-L1 cell line was roughly 100 fold higher than concentrations of glycerol in the media over 180 min from the isolated primary white adipocytes, and in the case of Iso+NaAc+Ins., the difference had increased to over 200 fold. From these studies it would appear that data collected from the 3T3-L1 cell line studies aligns with other published data (Taggart et al., 2005, Qiao et al., 2011), however, the concentration of both NEFA and glycerol in the media from isolated primary white adipocytes in both basal and stimulated state was not

similar to previously published data (Berggreen et al., 2009, Ge et al., 2008, Taggart et al., 2005). Any number of reasons may account for the results obtained within Chapter 6 including a problem with cell viability, or a low cell number in each treatment well.

In order to minimise mature primary adipocytes cell trauma during isolation, a selection of tissue DB were used to confirm the best combination of collagenase for the tissue type, and the resulting effects these buffers had on tissue viability (Fig 3.20). A combination of both adipocyte specific collagenase and liberase was chosen as the cells isolated under these conditions appeared to produce the greatest viability against cells isolated in either collagenase or liberase alone. However, as reported in Chapter 6, even after isolation with the preferred DB, mature primary adipocytes did not react to lipolytic stimulation with 5  $\mu$ M isoproterenol. This would suggest that during isolation the cells may have been stressed, contributing to the lack of change in NEFA and glycerol in the media. It may also be the case that the volume of cells used in the experiments was too low to detect a change in NEFA and glycerol. A packed cell volume of 150  $\mu$ l isolated mature adipocytes pooled from the tissue of 6 x 16 week old female mice was placed in 600  $\mu$ l culture media for sample collection. However, Rodbell (1964) reported the use of pooled adipose tissue from 3 rats (160-210g), with an estimated weight of 50 - 60 mg epididymal adipose tissue. Unfortunately, fat pads were not weighed in the experiments of this thesis, however, from a pilot study carried out 6 months prior to these studies, fat pads from the same age matched female mice were ~ 400 mg/fat pad, showing that tissue volume was not a limiting factor in these experiments. A release of 0.25  $\mu$ mol/hr NEFA reported by Taggart et al. (2005) was also much higher than that shown in the data in Chapter 6, however their study did not state the weight or method of normalisation of adipose tissue used per treatment in the study. Finally, the method of sample collection may be considered for revision if this experiment were to be repeated. The method of obtaining the correct treatment concentration by spiking the media instead of aspirating old media and replacing it could account for the lack of change in NEFA and glycerol. Cells were left to stabilize for 4 hr prior to the start of treatment incubation; however, instead of changing to treatment media at time 0 min, media were spiked with isoproterenol, acetate and insulin. As the cells were already releasing NEFA and glycerol into the media in the basal state during the 4 hr stabilization period, any accumulation in the media due to treatment conditions after time 0 may have been masked by the high concentration already present in the cell media. This may account for a lack of response in the basal

state however NEFA and glycerol release in the presence of the lipolytic stimulator isoproterenol would be expected to increase. A study has shown that there can be a 9 fold increase in glycerol release in wild type primary murine cells in the stimulated state (10  $\mu$ M isoproterenol) compared to basal state (Sztalryd et al., 2003), therefore, even with the lack of media change, we would still expect to see an increase in NEFA and/or glycerol concentration in the isoproterenol alone treated cells.

### **Future studies**

From data gathered and analysed throughout this thesis, several conclusion regarding the availability of acetate, and its effect on adipose tissue metabolism and function have been presented. It was evident in basal treatment conditions that NEFA in the media was reduced, and that the concentration of NEFA in the media at 180 min was lower than the concentration at 120 min (Fig. 4.1). This may be explained by re-uptake of LCFA from the media into the 3T3-L1 cells to be used in the re-esterification of NEFA. This observation is compounded by a lack of change in glycerol concentration into the medium compared to control. Data suggest that for the measurement of small changes in intracellular concentrations of TAG, the method used throughout these studies may not have had the required sensitivity. After contact with the manufacturer it was apparent that the Triglyceride kit had not been tested on adipose cells previously and so the manufacturer's instructions may not have been suitable for TAG analysis in lysed 3T3-L1 cells. Further searches of the literature show that an alternative method of organic extraction followed by colorimetric determination of TAG as described by Schwartz and Wolins (2007) (Ruiz-Gutiérrez and Pérez-Camino, 2000) may have been more relevant to the 3T3-L1 cell model, detecting micro molar concentration changes in TAG which would reflect small changes in the total release of NEFA and glycerol. Furthermore, if this technique had been employed, it may have been possible to identify whether increased acetate availability would have increased NEFA re-esterification in the basal and stimulated state, and hence lipogenesis. Future studies should therefore include investigation of markers of lipogenesis, including PPAR $\gamma$ .

In conclusion, for the first time to the best of our knowledge, acetate has been found to reduce the level of pHSL<sub>SER563</sub> in mature 3T3-L1 adipocytes. Secondly, in agreement with other groups, we have shown a decrease in concentration of NEFA in the media in the presence of a SCFA, however no significant change in glycerol. There is also data presented in this thesis to hypothesize a potential effect of increased acetate availability

on adipocyte re-esterification, as exemplified by the decrease in NEFA:glycerol ratio to 1.5:1. Although, further studies identifying the effect of sodium acetate on lipogenesis, through targets such as PPAR $\gamma$  would need to be investigated. This thesis also provides evidence that a concentration of  $\sim 10$   $\mu$ M isoproterenol is required to stimulate  $\beta$ -AR receptors by 100 % in 3T3-L1 mature adipocytes. Overall, the presence of increased acetate availability has been shown to have a small but significant effect on the lipolytic pathway in 3T3-L1 adipocytes via a reduction pHSL<sub>(SER563)</sub> activity and subsequent reduced NEFA concentration; however, these studies do not rule out a potential interaction between reduced lipolysis and lipogenesis regarding the mechanisms controlling the effect of SCFA on adipose tissue metabolism and function. Therefore, regarding physiological relevance to the whole organism, these data support further investigation of supra-physiological concentrations of acetate as a potential therapy in the treatment of dyslipidaemia, a common side-effect of type II diabetes. The lack of insulin sensitivity present in patients with type II diabetes, results in uninhibited activation of lipolysis and hence NEFA release (Bolinder et al., 1983, Choi et al., 2010, Berggreen et al., 2009). We have demonstrated via a lack of additive effect regarding pHSL that both acetate and insulin result in inhibition of the same pathway. The identification of GPR43 as a receptor for acetate, gives further merit to the therapeutic potential of increased acetate availability, by way of increasing the control of lipolysis via a separate receptor to IR, and ultimately reducing NEFA release. However, consideration to the limitations of these studies should also be considered. As mentioned previously, experiments with known inhibitors of GPR43 would confirm surface receptor activation by acetate and the downstream effect on pHSL. Similarly, the use of total HSL and other phosphorylation sites of HSL would be advantageous. This would give further support to the metabolic data in this study, which complement previously published data in the presence of increased SCFA availability.

## **Publications**

# Sodium acetate decreases phosphorylation of hormone sensitive lipase in isoproterenol-stimulated 3T3-L1 mature adipocytes

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**Keywords:** acetate, lipolysis, phosphorylation, hormone sensitive lipase, NEFA, glycerol

**Abbreviations:** BSA, bovine serum albumin; cAMP, 3'-5'-cyclic adenosine monophosphate; DMEM, Dulbecco modified eagle medium; NEFA, non-esterified fatty acid; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GLUT4, glucose transporter type 4; GPR-34, G-protein coupled receptor 43;  $\text{HCO}_3^-$ , bicarbonate; HSL<sub>(SER563)</sub>, hormone sensitive lipase; IR, insulin receptor; IRS1, insulin receptor substrate-1; NaAc, sodium acetate; NAD<sup>+</sup>, nicotinamide adenine dinucleotide; PDE3B, phosphodiesterase 3B; pHSL, phosphorylated hormone sensitive lipase; PI3K, phosphatidylinositol 3' kinase; PKA, protein kinase A; PKB, protein kinase B; SCFA, short-chain fatty acid

Lipolysis, the process of hydrolysis of stored triacylglycerol into glycerol and non-esterified fatty acids (NEFA), is reported to be reduced by short chain fatty acids (SCFA) but the mechanism of this inhibition is poorly understood. The aim of this study was to measure the phosphorylation at serine residue 563 of hormone sensitive lipase with and without exposure to sodium acetate. Using the 3T3-L1 cell line, we identified that stimulating the cells with isoproterenol increased phosphorylated hormone sensitive lipase (pHSL) expression by 60% compared with the basal state. In the presence of the SCFA acetate in stimulated cells, pHSL decreased by 15% compared with stimulated cells alone. These results were mirrored by the NEFA release from stimulated cells that had significantly decreased in the presence of sodium acetate after 60 min (from 0.53  $\mu\text{mol mg}^{-1}$  protein to 0.41  $\mu\text{mol mg}^{-1}$  protein, respectively,  $P = 0.004$ ); and 180 min (1.73  $\mu\text{mol mg}^{-1}$  protein to 1.13  $\mu\text{mol mg}^{-1}$  protein,  $P = 0.020$ ); however, treatment had no effect on glycerol release ( $P = 0.109$ ). In conclusion, exposure to 4 mM acetate reduced the level of phosphorylation of HSL<sub>(SER563)</sub> in mature 3T3-L1 adipocytes and lead to a significant reduction in NEFA release, although glycerol release was not affected.

## Introduction

Over the past 10 years there has been increased interest in the effect of nutritional substrates, such as short chain fatty acids (SCFA), on the metabolic pathway of lipolysis in white adipose tissue. Interest in surface receptor activation and changes to lipolysis, including glycerol and non-esterified fatty acid (NEFA) release have been reviewed in great detail.<sup>1,2</sup>

Renewed interest in the lipolytic pathway may stem from the findings that adipose tissue, far from being a deposition source for excess fat storage, is actually a multi-functional organ with an ability to generate and secrete adipokines that interact with the surrounding environment.<sup>3,4</sup> Within the mature adipocyte, lipid droplet formation and degradation is tightly controlled. There are many regulatory proteins and second messengers involved in the regulation of triacylglycerol hydrolysis, contributing to the complexity of lipolysis.<sup>5</sup> On the surface of mature adipocytes,  $\beta$ -adrenergic receptor stimulation results in an increase in release of glycerol and NEFA from stored triacylglycerol. The binding of ligands, e.g., epinephrine, norepinephrine, or glucagon, to these

surface receptors initiates a downstream reaction including activation of adenylyl cyclase, increased availability of cAMP, protein kinase A (PKA) phosphorylation of hormone sensitive lipase (pHSL) and perilipin, and subsequent hydrolysis of stored triacylglycerol into diacylglycerol, monoacylglycerol with the consequent release of NEFA and glycerol.<sup>6</sup>

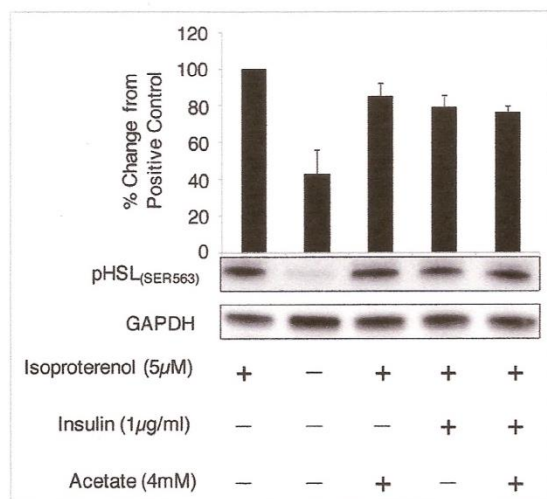
SCFAs can be generated through fermentation of fiber in the colon in humans, and also by the metabolism of alcohol in the liver.<sup>7,8</sup> SCFA are able to cross the gut lumen although the exact mechanism of membrane transport is still unknown. The presence of  $\text{HCO}_3^-$  or  $\text{Na}^+/\text{H}^+$  exchangers has been proposed<sup>9,10</sup> allowing the substrates including, acetate, propionate and butyrate, to become bound to serum albumin after entering the circulation. While the majority of butyrate (70–90%) and a proportion of propionate are metabolized through colonocyte oxidation, acetate and the remaining propionate are transported to the liver.<sup>11</sup> The majority of acetate absorbed by the gut and released by the liver is metabolized in mitochondria as an alternative energy source by surrounding tissues including the brain, smooth muscle, and heart.<sup>12,13</sup>

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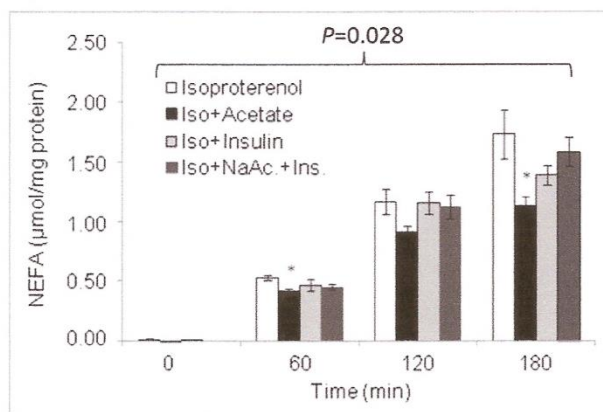
Submitted: 09/19/2013; Revised: 01/12/2014; Accepted: 01/21/2014

<http://dx.doi.org/10.4161/adip.27936>





**Figure 1.** Acetate and insulin reduces the phosphorylation of hormone sensitive lipase<sub>(SER563)</sub> in the presence of isoproterenol. Day 9 mature 3T3-L1 adipocytes were incubated with isoproterenol (5 µM) for 120 min, with or without insulin (1 µg/ml) and sodium acetate (4 mM). Cell lysates were analyzed by western blot and relative volume was calculated using ImageLab 3.0 normalizing to GAPDH (n = 4).



**Figure 2.** Temporal pattern of NEFA release following isoproterenol stimulation, in the presence of acetate, insulin, and NaAc.+Ins. Values are mean  $\pm$  SE, \* $P < 0.05$  vs. isoproterenol.  $P = 0.028$  indicates an increase in NEFA over time.  $n = 3-8$ .

An inhibitory G protein-coupled receptor (GPR43) for SCFA has been identified in white adipose tissue.<sup>14,15</sup> Reasons for the presence of a specific receptor for SCFA within white adipose tissue have not been fully identified, but it does suggest that SCFA may play a part in regulation of adipocyte lipolysis. A previous in vivo human study<sup>16</sup> observed a lack of glycerol and NEFA release into blood after the consumption of sodium acetate. The study aimed to identify the effects of acetate metabolism on fat and carbohydrate utilization. The authors administered either sodium acetate or sodium bicarbonate, both of which induced a metabolic alkalosis, where it was observed that both NEFA and glycerol

release increased in the first hour after sodium bicarbonate ingestion but that sodium acetate ingestion did not result in a change in NEFA and glycerol and there was a concomitant decrease in fat oxidation. The authors concluded that although acetate appears to have little effect on the basal rate of lipolysis it did suppress lipolysis during a metabolic alkalosis, i.e., conditions that stimulated lipolysis.<sup>16</sup> The present study therefore aims to identify whether sodium acetate affects adipose tissue lipolysis in vitro. By utilizing the murine cell line 3T3-L1, it was possible to monitor changes in phosphorylation of HSL<sub>(SER563)</sub>, the enzyme responsible for diacylglycerol hydrolysis, and identify the effect of sodium acetate on adipose cell lipolysis, as a measure of glycerol and NEFA and we hypothesized that in the presence of acetate the rate of NEFA and glycerol release would be reduced in isoproterenol stimulated cells.

## Results

### Changes in the level of phosphorylated hormone sensitive lipase in control and treated 3T3-L1 adipocytes

In order to identify if sodium acetate and insulin were acting within the lipolytic cascade, changes in the phosphorylation of HSL<sub>(SER563)</sub> were analyzed. In the presence of isoproterenol (Iso) the relative volume of pHS<sub>L</sub>(SER563) increased 60% compared with the basal state of pHS<sub>L</sub>(SER563), i.e., without stimulation (Fig. 1). Incubating the cells with the addition of Iso+acetate resulted in the relative volume of phosphorylated HSL<sub>(SER563)</sub> being reduced by 15% compared with isoproterenol stimulation alone. pHS<sub>L</sub> was also reduced in the presence of Iso+insulin by 21% and Iso+NaAc.+Ins. by 24% compared with isoproterenol stimulation.

### Changes in the metabolic response to control and treated 3T3-L1 cells in the presence of $\beta$ -adrenergic stimulation

The mature 3T3-L1 adipocytes were stimulated with a half-maximal dose of isoproterenol<sup>17</sup> over a period of 180 min and this resulted in a significant increase lipolytic rate as reflected by the release of both NEFA ( $P = 0.028$ ) and glycerol ( $P = 0.049$ ) over time when compared with the initial time point (see Figs. 2 and 3). As shown in Figure 2, in the presence of  $\beta$ -adrenergic receptor-stimulated lipolysis, there was a significant reduction in the cumulative release of NEFA in the presence of sodium acetate at both 60 min and 180 min ( $P = 0.004$  and  $P = 0.02$ , respectively) when compared with isoproterenol treatment alone. Iso+acetate treatment tended to reduce NEFA release at 120 min compared with isoproterenol treatment alone, although this was not statistically significant ( $P = 0.078$ ).

Between 0 and 60 min, the delta release of NEFA in Iso+acetate was  $0.413 \pm 0.02$   $\mu\text{mol mg}^{-1}$  protein 60 min<sup>-1</sup> and was found to be significantly lower ( $P = 0.017$ ) than that with isoproterenol alone ( $0.524 \pm 0.02$   $\mu\text{mol mg}^{-1}$  protein 60 min<sup>-1</sup>). The delta release of NEFA during Iso+insulin ( $0.458 \pm 0.04$   $\mu\text{mol mg}^{-1}$  protein 60 min<sup>-1</sup>) and Iso+NaAc.+Ins. ( $0.455 \pm 0.03$   $\mu\text{mol mg}^{-1}$  protein 60 min<sup>-1</sup>) treated cells also tended to be lower than that of isoproterenol alone; however these did not reach statistical significance ( $P = 0.375$  and  $P = 0.326$ , respectively). Over the next 60 min (60–120 min) the delta NEFA release did not differ between any treatment conditions. Over the final hour (120–180



min), delta NEFA release in the Iso+NaAc+Ins treated cells was significantly higher ( $P = 0.014$ ) compared with isoproterenol alone but treatment with either Iso+acetate and Iso+insulin did not result in any significant change.

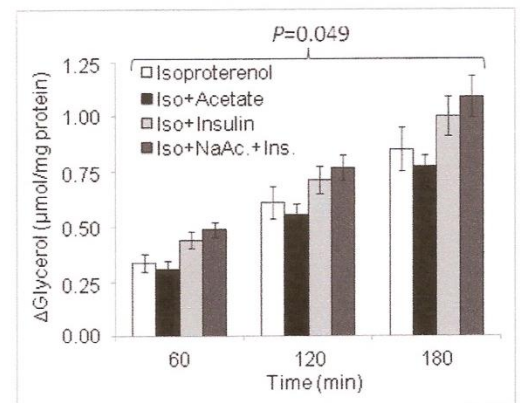
In the presence of each treatment there was no significant reduction in the change of glycerol concentration compared with isoproterenol alone. Although glycerol concentration appeared to increase at each time point it was only in the Iso+acetate treatment where glycerol release was observed to increase between 60 to 120 min ( $P = 0.006$ ) and from 120 to 180 min ( $P = 0.013$ ). In neither the Iso+insulin treatment nor Iso+NaAc+Ins, did we observe any change in glycerol concentration between 60 and 120 min. However, during the final 60 min the glycerol concentration increased significantly with Iso+insulin ( $P = 0.053$ ) and Iso+NaAc+Ins ( $P = 0.034$ ).

As both NEFA and glycerol samples were taken from the same well for each treatment, we expected a 3:1 ratio; however this was found not to be the case. At 120 and 180 min the ratio between NEFA release and glycerol release was ~1.5:1, i.e., half the release of NEFA that would have been expected.

## Discussion

The aim of this study was to identify whether sodium acetate affects adipose tissue lipolysis in vitro. These data demonstrate that an increase in acetate availability can cause a reduction in the phosphorylation of HSL at the serine 563 residue and supports the significant reduction in NEFA release that was observed in these cells but not our hypothesis regarding the effects on glycerol release.

The process of lipolysis is undoubtedly complex with many factors influencing this pathway; these include activation of inhibitory/stimulatory G protein-coupled receptors,  $\beta$ -adrenergic stimulation, insulin/glucagon signaling, and the interactions between these factors. The regulation of lipolysis is therefore tightly controlled but also depends on the energy state of the cell and the availability of substrates and hormones, e.g., glucose and insulin. In this study we were able to identify an intracellular change to lipid metabolism, from the basal to stimulated state, through examination of the phosphorylation status of HSL. Previously it has been shown that the phosphorylation status of HSL is critical in the catabolism of triacylglycerol. Using both an in vitro model and intact cell model Fredrikson et al.<sup>18</sup> demonstrated that the phosphorylation of HSL was associated with the availability of cAMP and that the enzyme activity of HSL increased 2-fold in the presence of the catalytic subunit of protein kinase and cAMP. Under basal conditions of lipolysis, there was continuous low level phosphorylation of HSL<sub>(563)</sub>. Upon stimulation with the  $\beta$ -adrenergic receptor agonist isoproterenol, the level of phosphorylation increased 60% and this observation supports previous work showing an increase in pHSL in the presence of isoproterenol<sup>19</sup> and similar increases in glycerol release in the presence of isoproterenol.<sup>18</sup> In this study we further demonstrated that the presence of sodium acetate reduced the phosphorylation of HSL by 15% compared with isoproterenol stimulated cells alone. It is clear that there are other serine residues (for example



**Figure 3.** Temporal pattern of glycerol release following Isoproterenol stimulation, in the presence of acetate, insulin, and NaAc+Ins. Values are mean  $\pm$  SE, \* $P < 0.05$  vs. isoproterenol.  $P = 0.049$  indicates an increase in glycerol over time.  $n = 3-6$ .

659 and 660) that are also linked to the activity of HSL.<sup>20,21</sup> and that future work should consider examining the phosphorylation status of these serine residues. In addition, the effects of acetate on the phosphorylation of serine residue 565 is also warranted because phosphorylation at this site is linked to the inhibition of HSL activity<sup>22</sup> and hence it might increase phosphorylation in a reciprocal manner to the decrease in HSL<sub>(563)</sub>. To the best of our knowledge these novel findings demonstrate the ability of the short chain fatty acid, acetate, to decrease the level of activity of one of the key enzymes, HSL, in the lipolytic pathway.

The most common endogenous inhibitor of the lipolytic pathway is insulin and activation of the insulin receptor (IR) and insulin receptor substrate-1 (IRS-1) is known to interact with the lipolytic pathway in adipocytes. For a review see reference 2. It is well documented that under conditions of excess glucose availability, insulin not only increases glucose uptake through the GLUT4 transporter<sup>19</sup> but also stimulates the phosphatidylinositol 3' kinase (PI3K) pathway.<sup>23,24</sup> The subsequent downstream signaling process is unresolved regarding the mechanism by which insulin decreases adipocyte lipolysis; however, protein kinase B (PKB) dependent<sup>25</sup> and -independent pathways<sup>23</sup> have been proposed to operate. Under conditions of high lipolytic stimulation, the phosphorylation of PKB on Ser<sub>273</sub> initiates the activation of phosphodiesterase 3B (PDE3B), which in turn hydrolyses cAMP into 5'-AMP.<sup>26</sup> This conversion to 5'-AMP results in a decrease in available cAMP and therefore reduces the ability of PKA to phosphorylate HSL and perilipin, both of which are essential for hydrolysis of stored triacylglycerol.<sup>27</sup> Our results support these findings since in the presence of 1.34  $\mu$ M insulin, there was an observed 21% reduction in pHSL compared with isoproterenol stimulated pHSL alone (Fig. 1). There was no evidence for an additive effect in the presence of both acetate and insulin in the stimulated cells as pHSL<sub>(SER563)</sub> had only decreased by 24% compared with isoproterenol stimulated cells.

Short chain fatty acids have an affinity to G protein-coupled receptors with acetate exhibiting specific affinity for GPR-43.<sup>15</sup>



Other studies have shown that activation of G protein-coupled receptors present in the cell membrane of adipocytes results in a reduction in the lipolytic rate as determined by the release of NEFA and/or glycerol.<sup>14,28-31</sup> In this study we have demonstrated that incubating mature 3T3-L1 adipocytes with supra-physiological levels of sodium acetate also reduces the rate of lipolysis, as measured by the rate of NEFA release, although glycerol release appeared to remain unaffected in the presence of sodium acetate. These data agree with previous reports showing changes in lipolysis, expressed as a reduction in the level of glycerol and/or NEFA release, in the presence of ligands of G protein-coupled receptors containing a small carboxylic acid tail region<sup>32,33</sup> similar to that of sodium acetate. The relationship between NEFA and glycerol release was also found to be lower than expected. It may, however, be that re-esterification of fatty acids into the adipocyte and an increase in mitochondrial oxidation within adipocytes had taken place, which would account for the reduction in NEFA to glycerol ratio.

In conclusion, these data support our hypothesis that increased availability of the short chain fatty acid, acetate, reduces the level of phosphorylation of HSL<sub>(SER563)</sub> in mature 3T3-L1 adipocytes, and lead to a significant reduction in NEFA release, but not of glycerol, into the surrounding environment.

## Materials and Methods

### Cell culture

Mature 3T3-L1 cells were cultured in a 12-well plate format for all experiments. Un-differentiated 3T3-L1 cells were plated in PM-1-L1 media (Zen Bio) and fed every 2 d until they reached confluence. The cells were left for a further 48 h to initiate growth arrest before the media was changed to DM-2-L1 differentiation media (Zen Bio). Cells were incubated in DM-2-L1 for 72 h before the media was gradually changed to AM-1-L1 media (Zen Bio), in accordance with the feeding manual published by Zen Bio. Cells were fed every other day until day 9 post-differentiation when cells were subjected to the various experimental treatments. Cells were treated in phenol red-free high glucose (4.5 g/L) DMEM (Sigma Aldrich) supplemented with 2% fatty acid free BSA (PAA Laboratories), subsequently referred to as treatment media. For cells that were treated with sodium acetate (Sigma Aldrich), 4 mM sodium acetate was added to the culture media 30 min prior to the start of lipolysis analysis, and was present in treatment media for each subsequent time point. After the 30 min pre-incubation, cells were washed in 37 °C 1× PBS and treated with or without 5 μM isoproterenol, 4 mM sodium acetate (Iso+acetate), 1.34 μmol insulin (Iso+insulin) (Invitrogen) or both acetate plus insulin (Iso+NaAc+Ins). The half-maximal dose of 5 μmol as a stimulating dose of isoproterenol was determined in a separate study (unpublished results) using a titration of isoproterenol against NEFA release in 3T3-L1 cells at the same stage of differentiation as the present study.

### Metabolic analysis

Cells were incubated for a total of 180 min in treatment media. Every 60 min, samples were collected from each treatment media and analyzed for both NEFA and glycerol concentration. Samples

were analyzed for NEFA using WAKO NEFA-HR(2) kit according to the manufacturer's instructions (Alpha Laboratories) and also for glycerol concentration using the method by Boobis and Maughan.<sup>34</sup> Briefly, stock solutions of the reaction mixture were prepared, including glycerol buffer, glycerol dehydrogenase, and NAD<sup>+</sup> (Sigma Aldrich), standards were prepared from a stock solution of 2 mM glycerol (Sigma Aldrich). Samples and standards were dispensed into a 96 plate and reaction mixture was added to each well. The plate was mixed well and left to incubate for 4 h. The reaction was stopped by the addition of sterile distilled H<sub>2</sub>O before the plate was read fluorometrically at excitation 340 nm and emission 460 nm, on a SpectraMax M5 microplate reader. Samples not analyzed on the same day were stored at -20 °C. NEFA and glycerol samples were normalized to total protein.<sup>35</sup>

### Western blotting

Cells were cultured and treated in a 12-well plate format. At the end of the 120 min incubation, cells were washed twice with ice cold 1× PBS, before the addition of lysis buffer (Cell Signaling Technology supplied by New England Biolabs) supplemented with 1 mM phenylmethylsulfonyl fluoride (Sigma Aldrich). Cells were incubated on ice for 5 min, and then scraped into suspension. Lysates were sonicated briefly in 1 s bursts for 10 s, and then centrifuged at 10 000 × g for 10 min at 4 °C. Due to the presence of free triacylglycerol, supernatants were aspirated and centrifuged for a second time. Protein concentration was calculated using the Bradford method, using BSA as the standard.<sup>35</sup> Protein samples of 15 or 25 μg were then separated by 4–15% SDS-PAGE and transferred to a polyvinylidene difluoride membrane (GE Healthcare). Each membrane was blocked overnight in 5% skimmed milk and then incubated with pHSL<sub>(SER563)</sub> and GAPDH primary antibodies (1:1000) (New England Biolabs), followed by horseradish peroxidase conjugated secondary IgG (1:3000) (New England Biolabs). Visualization of immunoreactive proteins occurred using the chemiluminescence detection kit LumiGLO with peroxide reagent (New England Biolabs). Images were acquired using ChemiDoc XRS+ and analyzed using ImageLab 3.0 software. First, the relative volumes of both pHSL and GAPDH were calculated before subtracting for background. The volume of pHSL and GAPDH was initially measured by determining the number of pixels within a standardized area for each sample from the recorded western blot image. Using isoproterenol as the positive control and standardizing the loading against GAPDH the remaining samples were standardized against the internal control. Furthermore the pHSL from the positive control was normalized against GAPDH and expressed as 100% from which all the remaining samples were expressed as a percentage of this maximum stimulated condition.

### Statistical analysis

All experiments were performed at least three times in triplicate. Statistical analysis was performed using SPSS Statistics 20 software. All data were analyzed for normality using the Shapiro–Wilks test. The glycerol Delta-glycerol and Delta NEFA data were found to be normally distributed thus analyzed using a two-way ANOVA followed by post-hoc Tukey test. In contrast the NEFA data were not normally distributed and thus



were analyzed using a Kruskal–Wallis test followed by a pair-wise Mann–Whitney. A *P* value of less than 0.05 was considered statistically significant.

#### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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